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PRINCIPAL INVESTIGATOR: John M. Ruppert, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294-3300

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John M. Ruppert, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35294-3300

E-Mail: mruppert@uab.edu

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13. ABSTRACT ***(Maximum 200 Words)***

A cell line termed RK3E provides a novel approach to identification and functional analysis of carcinoma-derived transforming oncogenes. Derived by immortalization of primary rat kidney cells, RK3E exhibit multiple properties of epithelia and specifically detect the tranforming activity a small subset of all oncogenes. The Aim of this proposal was to identify novel transforming activities in prostatic carcinoma by expression cloning in RK3E cells. This was not completed, in part due to routine technical problems encountered during cDNA library construction. Instead, we analyzed expression of GKLF, recently identified as a novel RK3E transforming oncogene, in several human tumor types. GKLF was found to be expressed at elevated levels in oral squamous cell carcinoma (100%) and in breast carcinoma (70%) *in vivo*. However, expression was reduced or unaltered in prostatic carcinoma. Thus, these results appear to exclude a role for GKLF in progression of prostatic carcinoma. The results suggest that carcinoma types for which oncogenes are poorly characterized, including prostatic carcinoma, can be rapidly assessed for novel transforming activities, and that genes identified by this *in vitro* assay are likely to be activated during tumor progression *in vivo*.

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Introduction:

Transforming oncogenes have traditionally been identified by introduction of tumor DNA or cDNA into mesenchymal cells such as NIH3T3. We have developed an expression cloning assay using an epithelial cell line, termed RK3E, that serves to identify oncogenes expressed in carcinomas in a sensitive and specific fashion. These cells form foci when transduced with activated RAS, activated β-catenin, or wild type alleles of plakoglobin, c-MYC, GLI, or GKLF (gut-enriched Kruppel-like factor, also known as KLF-4). In contrast, NIH3T3 cells form foci in response to RAS, but exhibit no response to any of the other major carcinoma oncogenes listed above. As few oncogenes are known to be activated in prostate cancer, we proposed to apply the RK3E assay to human prostate tumor cell lines. By analogy with a recently completed and published screen of breast and oral cancer lines, we expect that this approach will identify c-MYC as well as other known or novel transforming activities.

Body:

Prostatic carcinoma cells lines DU-145, PC-3, and LanCaP were purchased from the American Type Culture Collection, and passaged in tissue culture. Total RNA was extracted from exponentially growing cells and purified by guanidinium isothiocyanate acid phenol extraction (1). Comparable yields and purity were ascertained by absorption of ultraviolet light (see Table 1). As described in the Methods section of reference 2 (Appendix), equal quantities of total mRNA from each line was mixed together. Polyadenylated (Poly A+) mRNA in the mixture of total RNA was purified by two passages over oligo dT cellulose, quantified by absorption of ultraviolet light, and stored at –70C.

Table 1: Preparation of mRNA from prostate cancer cell lines.

Cell line	RNA yield (μg/ml)	Optical density (260nm/280nm)
LNCaP	197	2.02
DU145	79.4	1.98
PC-3	150	1.97

A cDNA synthesis kit was purchased from Stratagene (La Jolla, CA) and used to prepare first strand and second strand cDNA using a trace amount of radiolabel to facilitate visualization of the final product. Gel electrophoresis of this cDNA indicated a low final yield of cDNA, as determined by ethidium bromide staining and autoradiography of the dried gel. Rather than perform the screen using sub-optimal yields of cDNA, we elected to repeat the RNA extraction, poly A+ mRNA isolation, and cDNA synthesis. This work was not completed owing to an increased focus upon a novel oncogene recently isolated by the same assay (2).

In the final 18 months of the funding period, the laboratory's work became more focussed upon GKLF. Specifically, we found that this novel oncogene is activated at the level of mRNA and protein expression in 70% of breast cancers (3). We also initiated

new studies to demonstrate the transforming activity of this gene in transgenic mouse skin and in the mouse breast. This preliminary work is in progress at the time of the annual report and we anticipate publication of the work later this year.

To analyze GKLF expression *in vivo*, we worked out the technique of mRNA *in situ* hybridization and used the assay to analyze expression of GKLF during progression of multiple tumor types including oral squamous cell carcinoma, breast cancer, prostate cancer, and colorectal carcinoma (2,3). In brief, GKLF is upregulated during progression of 70% of breast tumors and in 100% or oral squamous cell carcinomas. Although GKLF is expressed in normal prostate and colorectal epithelium, expression is reduced or unchanged during tumor progression in these tissues.

A novel monoclonal antibody was raised in collaboration with J.E. Engler, Dept. of Biochemistry and Molecular Genetics, University of Alabama at Birmingham. Termed clone IE5, this antibody specifically detects GKLF by immunohistochemical analysis of human tissues (3). We used this reagent to perform immunohistochemical analysis of the polypeptide, and were albe to confirm the mRNA in situ hybridization studies.

Therefore, these studies identify breast carcinoma and squamous cell carcinoma as tumor-types in which GKLF activation may be important, and appear to exclude involvement in some other tumor types such as prostate cancer and colorectal cancer.

The work we have performed to date deviates significantly from the original Statement of Work. As a new investigator, I underestimated the degree to which trainees in the laboratory would be reliant upon me for successful completion of their lab work. I had anticipated that my detailed protocol of the cDNA library construction and of the expression cloning screen would enable a graduate student to perform the work, and that my involvement would be limited to daily advice and troubleshooting. In reality, only a few of my trainees have exhibited the independence, self-motivation, and work ethic necessary for success.

I was not able to perform the screen myself. In 1998-99, I found it necessary to work desperately to generate enough preliminary data to renew our only other grant, NIH R29 CA65686, without which the lab would have closed and my job would have ended. We were successful in identifying GKLF/KLF4 as an oncogene, characterizing its expression in human tumors, and generating a mouse model. These results were sufficient to renew our funding at the NIH, and we also competed successfully for a Breast SPORE at UAB.

The Tasks listed under the SOW were nearly all "Not Initiated" because we had technical problems synthesizing adequate cDNA. As a result, our results to date are of questionable relevance to prostate cancer.

While I regret not having completed the work as proposed – it represents my biggest failure as a faculty member - the data we have generated on KLF4 supports the validity of the IDEA Award project, as does our demonstration that KLF4 is not up-regulated in Prostate cancer. I do plan to perform the screen in the lab as described, as the idea seems better than ever, and will offer that project to a postdoctoral fellow to be recruited this year. At this time we have the resources to support such an effort.

Key Research Accomplishments:

- Culture and passage of prostate cancer cell lines
- Preparation of total mRNA
- Isolation of polyA+ mRNA
- Synthesis of cDNA
- Validation of the RK3E assay by showing that GKLF is an oncogene activated during carcinoma progression *in vivo* (breast carcinoma, oral squamous cell carcinoma)
- Development of an immunohistochemical assay for human GKLF polypeptide
- GKLF expression is not up-regulated during prostate cancer progression
- GKLF mRNA and protein is induced in 70% of human breast cancers or ductal carcinoma in situ (DCIS)
- GKLF mRNA and protein is induced in dysplastic oral epithelium or carcinoma of the oro-pharynx

Reportable outcomes:

Provisional patent application, May 19, 1999: Oncogene Identification by Transformation of RK3E cells and uses thereof; D6236

Foster, K. Wade, Songrong Ren, Iuri D. Louro, Susan M. Lobo-Ruppert, Peggy McKie-Bell, William Grizzle, Martha R. Hayes, Thomas R. Broker, Louise T. Chow, and **J. Michael Ruppert**. 1999. Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. Cell Growth & Differentiation 10: 423-434.

Foster, K. Wade, Andra R. Frost, Peggy McKie-Bell, Chin-Yu Lin, Jeffrey A. Engler, William E. Grizzle, and **J. Michael Ruppert**. 2000. Increase of GKLF messenger RNA and protein expression during progression of breast cancer. Cancer Research 60: 6488-6495.

PhD to K. Wade Foster, to graduate in Summer of 2001.

An NIH grant, RO1 CA65686-06, was obtained to further study GKLF in breast and oral cancer.

Conclusions:

The ability to identify the major transforming oncogenes activated in epithelial-derived tumors is critical to the understanding carcinoma pathogenesis. We found that transformation of epithelial cells *in vitro* is a highly specific assay that does not lead to identification of artifacts such as truncated cDNAs or cDNAs that are irrelevant to tumor progression *in vivo*. Identification of GKLF as an oncogene suggests that other major transforming activities in human tumors may be similarly unrecognized. Although activated in breast and oral cancer, this gene does not appear to play a role in tumors of the prostate or colon.

Significance/So What: Exclusion of a role for GKLF in prostatic carcinoma is significant in that none of the oncogenes currently known to transform RK3E cells are known by us to be consistently activated in this tumor type. In contrast, tumors of the breast, oral cancer, colon cancer and skin cancer exhibit frequent activation of a member of this class of oncogenes. Although we have not successfully completed a screen in prostate cancer as originally proposed, we believe that application of the assay to this poorly understood tumor-type may well lead to better insight into pathogenesis, and hope to complete such a screen in the future.

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1. Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162*: 156-159, 1987.

2. Foster, K. Wade, Songrong Ren, Iuri D. Louro, Susan M. Lobo-Ruppert, Peggy McKie-Bell, William Grizzle, Martha R. Hayes, Thomas R. Broker, Louise T. Chow, and **J. Michael Ruppert**. 1999. Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. Cell Growth & Differentiation 10: 423-434.

3. Foster, K. Wade, Andra R. Frost, Peggy McKie-Bell, Chin-Yu Lin, Jeffrey A. Engler, William E. Grizzle, and **J. Michael Ruppert**. 2000. Increase of GKLF messenger RNA and protein expression during progression of breast cancer. Cancer Research 60: 6488-6495.

Appendices:

Reprints of references shown in Bibliography (below).

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1. Foster, K. Wade, Songrong Ren, Iuri D. Louro, Susan M. Lobo-Ruppert, Peggy McKie-Bell, William Grizzle, Martha R. Hayes, Thomas R. Broker, Louise T. Chow, and **J. Michael Ruppert**. 1999. Oncogene expression cloning by retroviral transduction of adenovirus E1A-immortalized rat kidney RK3E cells: transformation of a host with epithelial features by c-MYC and the zinc finger protein GKLF. Cell Growth & Differentiation 10: 423-434.

2. Foster, K. Wade, Andra R. Frost, Peggy McKie-Bell, Chin-Yu Lin, Jeffrey A. Engler, William E. Grizzle, and **J. Michael Ruppert**. 2000. Increase of GKLF messenger RNA and protein expression during progression of breast cancer. Cancer Research 60: 6488-6495.

Personnel:

Persons receiving pay from the research effort include research assistant P. McKie-Bell, and principal investigator J. Michael Ruppert. Graduate student W. Foster is paid by the Division of Hematology-Oncology as part of a matching agreement with the Army.

[CANCER RESEARCH 60, 6488–6495, November 15, 2000]

Increase of GKLF Messenger RNA and Protein Expression during Progression of Breast Cancer[1]

K. Wade Foster, Andra R. Frost, Peggy McKie-Bell, Chin-Yu Lin, Jeffrey A. Engler, William E. Grizzle, and J. Michael Ruppert[2]

Department of Biochemistry and Molecular Genetics [K. W. F., J. A. E., J. M. R.], Department of Pathology [A. R. F., W. E. G.], Division of Hematology/Oncology, Department of Medicine [P. M-B., J. M. R.], Medical Statistics Section, Department of Medicine [C-Y. L.], and Oral Cancer Research Center and Comprehensive Cancer Center [J. A. E., W. E. G., J. M. R.], University of Alabama at Birmingham School of Medicine, Birmingham, Alabama 35294-3300

ABSTRACT

Genetic alterations found in carcinomas can alter specific regulatory pathways and provide a selective growth advantage by activation of transforming oncogenes. A subset of these genes, including wild-type alleles of *GLI* or *c-MYC*, and activated alleles of *RAS* or *β-catenin*, exhibit transforming activity when expressed in diploid epithelial RK3E cells *in vitro*. By *in vitro* transformation of these cells, the zinc finger protein GKLF/KLF-4 was recently identified as a novel oncogene. Although GKLF is normally expressed in superficial, differentiating epithelial cells of the skin, oral mucosa, and gut, expression is consistently up-regulated in dysplastic epithelium and in squamous cell carcinoma of the oral cavity. In the current study, we used *in situ* hybridization, Northern blot analysis, and immunohistochemistry to detect GKLF at various stages of tumor progression in the breast, prostate, and colon. Overall, expression of GKLF mRNA was detected by *in situ* hybridization in 21 of 31 cases (68%) of carcinoma of the breast. Low-level expression of GKLF mRNA was observed in morphologically normal (uninvolved) breast epithelium adjacent to tumor cells. Increased expression was observed in neoplastic cells compared with adjacent uninvolved epithelium for 14 of 19 cases examined (74%). Ductal carcinoma *in situ* exhibited similar expression as invasive carcinoma, suggesting that GKLF is activated prior to invasion through the basement membrane. Expression as determined by Northern blot was increased in most breast tumor cell lines and in immortalized human mammary epithelial cells when these were compared with finite-life span human mammary epithelial cells. Alteration of GKLF expression was confirmed by the use of a novel monoclonal antibody that detected the protein in normal and neoplastic tissues in a distribution consistent with localization of the mRNA. In contrast to most breast tumors, expression of GKLF in tumor cells of colorectal or prostatic carcinomas was reduced or unaltered compared with normal epithelium. The results demonstrate that GKLF expression in epithelial compartments is altered in a tissue-type specific fashion during tumor progression, and suggest that increased expression of GKLF mRNA and protein may contribute to the malignant phenotype of breast tumors.

INTRODUCTION

Multiple physiological changes lead to acquisition of the malignant phenotype. These include self-sufficiency in growth signaling, insensitivity to growth-inhibitory signals, inhibition of apoptosis, immortalization, induction of angiogenesis, and the ability to invade and metastasize (1, 2). Genetic analyses of inherited predispositions to develop specific carcinomas enabled isolation of tumor suppressor genes important in both inherited and sporadic disease (3), and subsequent functional studies identified certain of these genes as regulators of classical transforming oncogenes. Thus, alterations in the tumor suppressor patched (PTC1), or in other molecules that transduce the hedgehog signal, result in activation of GLI mRNA expression in virtually all basal cell carcinomas of the skin (4–7). In contrast, alterations in the adenomatous polyposis coli (APC) pathway activate the β-catenin/TCF-4 complex and transcription of *c-MYC* and *cyclin D1* during colorectal tumor progression (8, 9). Modulation of the PTC1 and APC pathways by alteration of the mouse genome provides additional support of a role for these gene products in specific tumor types (10–14). Therefore, these pathways exhibit properties of a gatekeeper, indicating that alteration of the pathway in a specific tissue is rate-limiting for tumor progression, and that alterations are found in a large proportion of inherited as well as sporadic tumors (15, 16).

By expression cloning we recently identified the zinc finger protein GKLF[3] as a novel transforming oncogene when expressed in RK3E cells, a diploid epithelial cell line derived from primary rat kidney cells and immortalized with adenovirus E1A (17). cDNA libraries were prepared using mRNA from human oral squamous cell or breast carcinoma cell lines, tumor-types not reported to exhibit frequent genetic alterations that activate well-characterized oncogenes such as *RAS*, *GLI*, or *β-catenin* (18). Retroviral transduction of these libraries into RK3E cells induced morphologically transformed foci, 11 of which were subsequently attributed to enforced expression of wild-type human *c-MYC*. Two other transformed foci contained independently derived, wild-type alleles of *GKLF*. No other genes were identified in the screen, suggesting that only a select subset of all oncogenes are able to transform these cells.

Whereas enforced expression of a human wild-type *GKLF* transgene in RK3E cells induces morphological transformation *in vitro* and tumorigenicity in athymic mice, the doubling time of GKLF-expressing cells was considerably longer than for RK3E cells (27 h *versus* 12 h, respectively; Refs. 17, 19). Similar results were obtained for other oncogenes, including *GLI* and *c-MYC*, as cells expressing these genes exhibited doubling times of 18 and 19 h, respectively. These oncogenes may therefore function in epithelial cells by interfering specifically with contact inhibition rather than by inducing a more general increase in the rate of cell division.

In support of a role for *GKLF* as an oncogene, we observed increased expression of GKLF mRNA during progression of squamous cell carcinomas of the oropharynx. As demonstrated by mRNA ISH analysis of surgical specimens, expression in normal epithelial cells is limited to the differentiating compartment. Expression in dysplastic oral epithelium is increased overall and is found in all cell layers, and GKLF is expressed at similar levels in dysplasia and in invasive carcinoma. These results identified loss of the compartment-specific pattern of GKLF mRNA expression in epithelium as a candidate mechanism of tumor progression in oral cancer (17).

GKLF encodes a DNA-binding transcription factor with functional

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[2] To whom requests for reprints should be addressed, at Department of Medicine, Room 570 Wallace Tumor Institute, University of Alabama at Birmingham School of Medicine, Birmingham, AL 35294-3300. Phone: (205) 975-0556; Fax: (205) 934-9573; E-mail: mruppert@uab.edu.

[3] The abbreviations used are: GKLF, gut-enriched Krüppel-like factor; DCIS, ductal carcinoma *in situ*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMEC, human mammary epithelial cell; IDC, internal ductal carcinoma; ISH, mRNA *in situ* hybridization; SAGE, serial analysis of gene expression.

domains that mediate activation or repression of transcription (20–22). GKLF is essential for the barrier function of skin, because homozygous knockout mice exhibit morphologically normal skin but die postpartum due to dehydration (23). Transcriptional targets of GKLF that may be relevant to epithelial differentiation have been preliminarily identified (23–25). As shown by analysis of normal mouse or human tissues, GKLF is preferentially expressed in differentiating epithelial cells of the skin, gut, oral cavity, and thymus (17, 20, 23, 26, 27). In contrast to human oral squamous cell carcinoma, expression of GKLF mRNA was found to be reduced in mouse models of intestinal tumorigenesis or hyperproliferation (28, 29). Independently, analysis of human colorectal mucosa and tumors by SAGE confirmed the earlier studies in mice (30, 31). Specifically, mRNA from specimens of normal colonic mucosa generated GKLF tags at frequencies of 138 or 99 per million SAGE tags, whereas mRNA from a microdissected tumor generated only 20 tags per million. These results suggested that GKLF expression is regulated during neoplastic progression in a tumor type-specific fashion.

Increased expression of specific oncogenes in tumors can result from genetic alterations and play a causal role in tumor progression. Alternatively, expression of some oncogenes is cell cycle dependent, and increased expression can occur as a consequence of increased proliferation or altered cell cycle occupancy of tumor cells. In multiple normal tissues as well as in certain cell lines, GKLF expression is reduced in actively cycling cells compared with terminally differentiated or growth-arrested cells, and enforced expression of GKLF in cultured cell lines can retard cell cycle progression (26, 32). These properties predicted that GKLF expression might be reduced in tumors, as observed in colorectal carcinoma. Increased expression in other tumor-types is therefore somewhat unexpected and may result from specific alterations in the pathways that regulate GKLF transcription in normal cells.

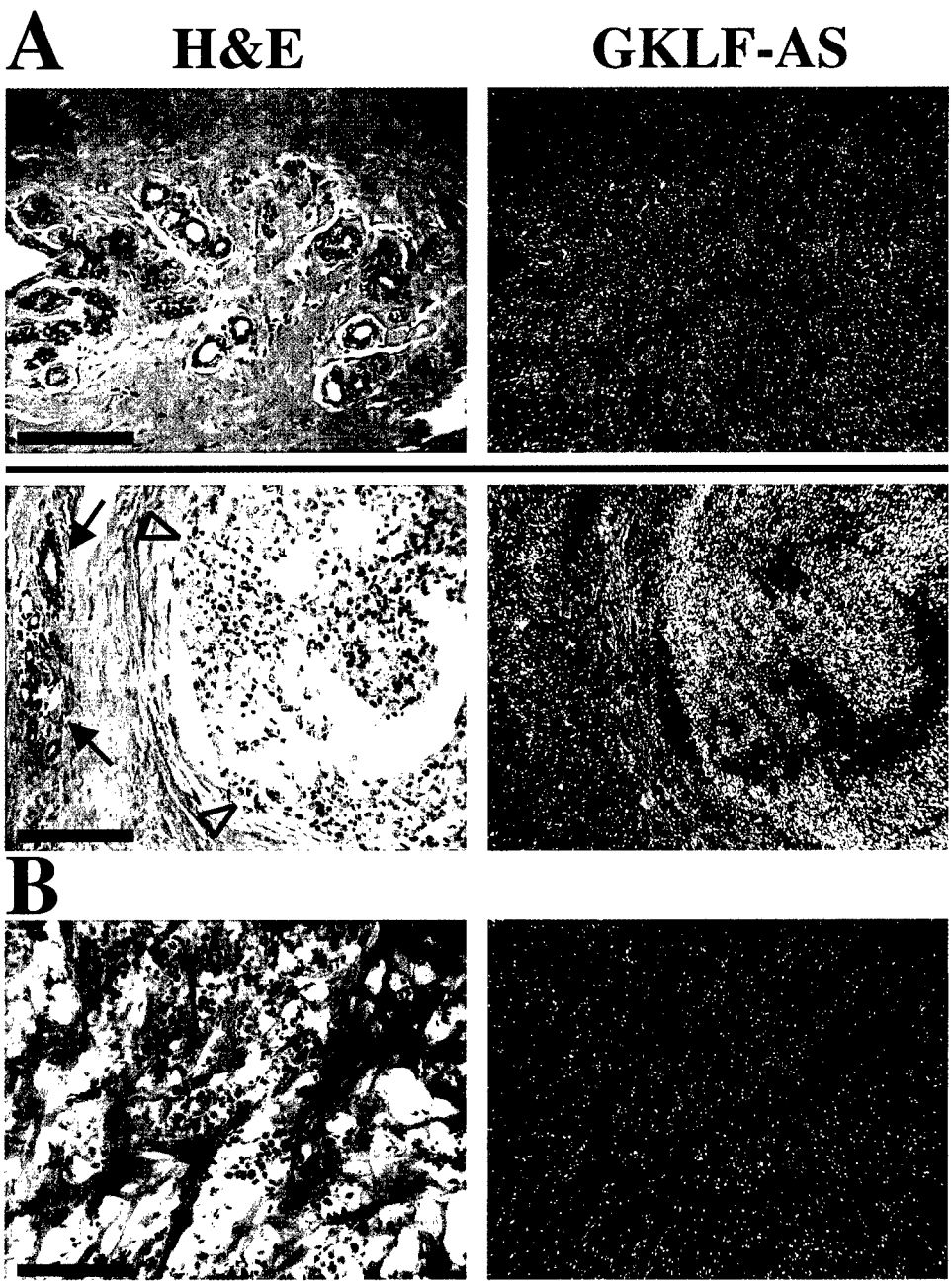

Fig. 1. ISH analysis of GKLF mRNA in carcinoma of the breast. Two distinct cases were analyzed by applying an antisense (*GKLF-AS*) [^{35}S]-labeled RNA probe to sections of paraffin-embedded (*A*) or fresh-frozen (*B*) surgical material. Brightfield (*left*) and darkfield (*right*) views are shown. Sections were stained with H&E. Hybridization to a sense control probe resulted in an average of 0.4 grains/nucleus (not shown). *A*, two areas of the same slide are shown, with uninvolved (*i.e.*, morphologically normal) breast epithelium (*upper plate*) adjacent to an area (*lower plate*) containing DCIS (*arrowheads*) and additional uninvolved tissue (*arrows*). *B*, IDC admixed with cords of stroma. Scale bars = 160 μm.

To better understand the spectrum of tumor-types that exhibit GKLF activation, we obtained samples of breast carcinoma, colorectal carcinoma, and prostatic carcinoma and analyzed expression of GKLF in malignant cells and in adjacent normal-appearing epithelium (*i.e.*, uninvolved epithelium). The results show that levels of GKLF mRNA and protein are each up-regulated before invasion in a majority of cases of breast cancer, but not in tumors of the colorectum or prostate. In neoplastic lesions of the breast as well as in cultured mammary epithelial cells *in vitro*, increased GKLF expression appears to precede overtly malignant behavior. The potent transforming activity of GKLF *in vitro*, the tumor type-specific activation of expression *in vivo*, and activation early during tumor progression identify this oncogene as a potential effector of tumor progression in the breast.

MATERIALS AND METHODS

Tissue Procurement. Fresh-frozen and paraffin-embedded samples were obtained through the Tissue Procurement Core Facility of the University of Alabama at Birmingham Comprehensive Cancer Center and through the Southern Division of the Cooperative Human Tissue Network.

Table 1 *mRNA ISH analysis of GKLF in tumors*[a]

Carcinoma of the breast

		GKLF-AS				
Case	PE/FF	U	D	T	GKLF-S	GAPDH-AS
1	FF	0.5	2.5	–	0.0	+
2	FF	–	–	2.0	0.0	+
3	FF	0.0	–	1.0	0.0	+
4	FF	–	–	0.0	0.0	+
5	FF	–	–	0.0	0.0	NT
6	FF	–	–	0.0	0.0	NT
7	FF	–	2.0	2.0	0.0	NT
8	FF	0.0	1.0	1.0	0.0	NT
9	FF	–	–	0.0	0.0	NT
10	FF	–	–	0.0	0.0	NT
11	FF	–	–	0.0	0.0	NT
12	FF	–	–	0.5	0.0	NT
13	FF	0.0	–	0.5	0.0	NT
14	FF	–	–	0.5	0.0	NT
15	PE	–	–	1.5	NT	+
16	PE	0.0	–	1.0	NT	+
17	PE	0.0	–	1.0	NT	+
18	PE	0.0	–	2.0	NT	+
19	PE	–	–	0.0	NT	+
20	PE	1.0	2.0	1.0	NT	+
21	PE	0.5	–	1.5	NT	+
22	PE	0.5	2.0	2.0	NT	+
23	PE	1.0	–	1.0	0.0	+
24	PE	0.5	1.0	1.2	0.0	+
25	PE	0.3	1.2	1.2	0.0	+
26	PE	0.5	1.5	1.5	0.0	+
27	PE	0.0	0.0	0.0	0.0	+
28	PE	0.0	0.0	0.0	0.0	+
29	PE	0.0	0.0	0.0	0.0	+
30	PE	0.5	1.0	1.0	0.0	+
31	PE	0.0	1.0	1.5	0.0	0.0

Carcinoma of the prostate

		GKLF-AS				
Case	PE/FF	U	PIN	T	GKLF-S	GAPDH-AS
1	PE	1.0	–	0.0	NT	+
2	PE	–	–	0.0	NT	+
3	PE	1.0	–	1.0	NT	+
4	PE	1.0	1.0	0.0	NT	0.0

[a] Results obtained for sense (*S*) or antisense (*AS*) probes are presented. Scoring of GKLF used a scale of 0.0–4.0 as described in "Materials and Methods," whereas GAPDH was scored as detected (+) or undetected (0.0). Numbers indicate the level of gene expression for histologically distinct tissue within the same section. A dash (–) indicates that no tissue in the section exhibited the specific histopathologic feature. *PE*, paraffin-embedded; *FF*, fresh-frozen; *U*, uninvolved or morphologically normal epithelium; *D*, ductal carcinoma *in situ*; *PIN*, prostatic intraepithelial neoplasia; *T*, invasive tumor cells; *NT*, not tested.

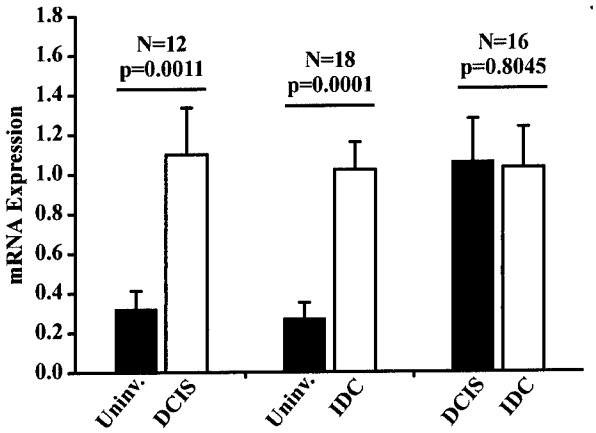

Fig. 2. GKLF mRNA expression in normal and neoplastic breast tissue. The data in Table 1 was analyzed using a paired *t* test. Sample size (*N*), statistical significance (*p*), and SE are indicated for each comparison. *Uninv.*, uninvolved ducts.

mRNA Expression. ISH was conducted as described (17) using sense and antisense [^{35}S]-labeled riboprobes prepared by *in vitro* transcription of a cDNA fragment corresponding to the 3′ untranslated region of human *GKLF*. A *GAPDH* antisense probe corresponding to bases 366–680 (GenBank accession no. M33197) was synthesized using a commercially available template (Ambion, Inc., Austin, TX). High stringency washes were in 0.1 × SSC and 0.1% (v/v) 2-mercaptoethanol at 58°C for *GKLF* or 68°C for *GAPDH*. Slides were coated with emulsion and exposed for 14 days. The number of silver grains/nucleus were counted within representative areas by two individuals, and a score from 0.0 to 4.0 was recorded. A score of 0.0 indicated only nonspecific background, as determined using the sense control, and 1.0 corresponded to an average of four grains/nucleus.

Breast adenocarcinoma cell lines were obtained from the American Type Culture Collection (Manassus, MD). HMECs were described previously and were cultured in mammary epithelial basal media (Clonetics Corp., Walkersville, MD; Ref. 33). Extracts were prepared from exponentially growing cells at 70% confluence, and total RNA isolation and Northern blot analysis were performed as described (17).

Isolation of an Anti-GKLF Monoclonal Antibody. The region of the human GKLF cDNA encoding bases 479-1197 (GenBank accession no. AF105036) was cloned into plasmid pET-32a and expressed in *Escherichia coli* BL21(DE3) bacteria as a histidine-tagged protein. Protein was purified from the bacteria after induction with isopropyl-1-thio-β-D-galactopyranoside using a His-Trap Ni-agarose column (Amersham Pharmacia Biotech, Piscataway, NJ) and eluted with 500 mM imidazole. Purified protein was used to immunize two mice, and lymphocytes were fused with murine myeloma cells (PX63-Ag8.653) as described previously (34). Hybridomas that were immunoreactive in an ELISA assay for the purified antigen were cloned and recloned by limiting dilution. Positive clones were identified by ELISA, and an IgG1 antibody (αGKLF) was purified from ascites on a protein A affinity column.

Immunohistochemistry. Tissues were fixed in neutral buffered formalin and embedded in paraffin. Deparaffinized tissue sections were incubated with αGKLF at a concentration of 1.0 μg/ml for 1 h at room temperature, and processed as described (35). Immunodetection was performed using a biotinylated secondary antibody, streptavidin-horseradish peroxidase detection system (Signet Laboratories, Dedham, MA), and the chromogenic substrate diaminobenzidine (Biogenex, San Ramon, CA). Sections were counterstained with hematoxylin. Results were scored by using a 0.0 to 4.0 scoring system where 4.0 corresponds to a saturated signal (36).

Statistical Analyses. Paired *t* tests were used to compare the differences in expression in breast epithelial cells at various stages of tumor progression (37). Pearson correlation coefficients were used to compare results obtained by ISH to those obtained for the same cases using immunohistochemistry.

RESULTS

GKLF mRNA Expression Is Up-Regulated during Breast Tumor Progression. Previously, SAGE analysis of purified normal breast epithelial cells detected GKLF transcripts at an abundance of

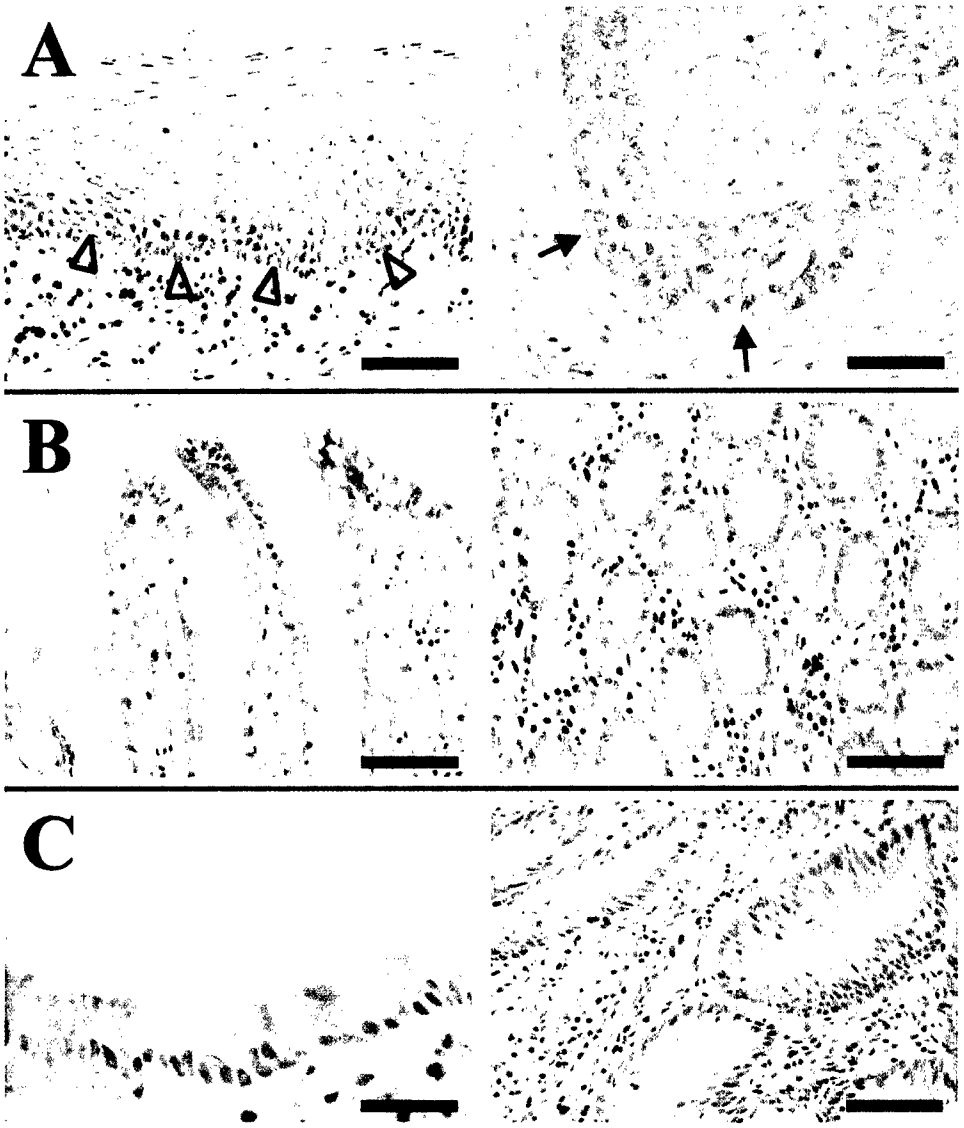

Fig. 3. Immunostaining of human tissues with αGKLF monoclonal antibody. Each panel (*A-C*) illustrates adjacent areas of a tissue section. *A*, uninvolved oral epithelium (*left*) and invasive oral squamous cell carcinoma (*right*). *Arrowheads* indicate the basal cell layer, whereas *arrows* indicate invasive carcinoma. Staining of tumor cells and of superficial epithelial cells is indicated by a *brown* precipitate. *B*, a section of small bowel illustrating increased staining of superficial epithelium (*left*) compared with cells deeper within crypts (*right*). *C*, a case of colorectal carcinoma, with increased staining of uninvolved superficial mucosa (*left*) compared with adjacent tumor cells (*right*). Scale bar for *C* (*left panel*) = 45 μm; other scale bars = 140 μm..

40 tags per million (31, 38), and Northern blot analysis of breast tumor cell lines revealed the presence of GKLF transcripts (17). Using sense and antisense [^{35}S]-labeled riboprobes, we examined the expression of GKLF mRNA in 31 cases of carcinoma of the breast. Specificity of hybridization was determined by using the sense probe as a negative control or by hybridization of the antisense probe to human foreskin, in which GKLF was specifically detected in suprabasal epithelial cells (not shown).

Expression of GKLF was detected in malignant cells in 21 of 31 cases of ductal adenocarcinoma (68%, Fig. 1, Table 1). For several cases that exhibited no detectable expression of GKLF, prominent expression of the housekeeping gene *GAPDH* was observed, indicating that overall mRNA integrity was maintained and that failure to identify GKLF transcripts may reflect reduced levels of expression. GKLF expression was increased in malignant cells of 14 of 19 cases that contained adjacent uninvolved epithelium (Fig. 1*A*). For 7 of these 14 cases, no specific signal was detected in adjacent uninvolved epithelium. In the other seven cases, expression was detected in both uninvolved and malignant cells, with expression of GKLF in malignant cells increased by 3- to 5-fold compared with uninvolved epithelium. Within tumors, expression of GKLF was specific to malignant cells, with little or no expression detected in stromal components (Fig. 1*B*).

GKLF expression in DCIS was not significantly different from invasive carcinoma, but expression in both lesions was higher than for uninvolved breast epithelium (Table 1, Fig. 2). In contrast to results obtained in breast tumors, examination of several cases of prostatic carcinoma revealed equal or reduced expression in tumor cells compared with adjacent uninvolved glandular epithelial cells (Table 1). In summary, the results suggest that GKLF mRNA expression is activated in approximately two-thirds of breast carcinomas, and that expression in positive cases is consistently induced in DCIS before invasion.

Characterization of a GKLF-specific Monoclonal Antibody. An IgG1 isotype antibody raised against bacterially expressed GKLF was subsequently referred to as αGKLF. Immunoblot analysis of GKLF-transformed RK3E cells and control cell lines detected a single protein species of 55 kDa consistent with the predicted size of the full-length polypeptide (data not shown). Compared with RK3E cells or control cell lines transformed by other oncogenes, apparent GKLF abundance was increased by several-fold in each of two cell lines transformed by the human expression vector. The epitope recognized by the antibody may be denaturation-sensitive, as a signal was obtained only after overnight exposure of autoradiographic film using a standard chemiluminescence protocol. The antibody was not sufficiently sensitive

Table 2 *Immunohistochemical analysis of GKLF in tumors*[a]

Carcinoma of the breast						
	Uninvolved		DCIS		Invasive tumor cells	
Case	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
23	0.25	0.45	–	–	0.35	0.55
24	0.50	1.30	1.00	1.30	1.00	1.30
25	0.65	0.95	0.45	1.40	0.38	1.35
26	0.18	0.75	0.03	1.20	0.12	1.05
27	0.10	1.30	0.00	1.10	0.05	0.50
28	0.10	0.30	–	–	0.35	0.20
29	0.00	0.00	0.10	0.75	0.05	0.75
30	0.00	0.20	0.10	1.05	–	–
31	0.00	0.10	0.65	0.65	0.70	1.15
32	0.25	0.55	0.55	0.75	0.42	0.85
33	0.80	0.45	–	–	0.50	1.25
34	0.18	0.50	–	–	0.45	1.15
35	0.30	0.35	0.60	1.60	0.65	1.50
36	0.00	0.05	0.55	1.70	0.75	1.00
37	0.70	0.60	–	–	1.65	1.80
38	–	–	0.00	0.90	0.00	1.50
39	0.55	0.70	0.75	0.85	1.75	1.75
40	0.35	0.50	0.75	0.90	0.75	0.85

Colorectal carcinoma						
	Normal Superficial[b]		Normal Deep[c]		Tumor[d]	
Case	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
1	0.45	1.00	0.25	0.05	0.00	0.85
2	0.40	0.60	0.40	0.25	0.20	0.35
3	0.15	1.15	0.30	0.80	0.25	0.85
4	0.00	1.30	0.00	0.15	0.00	0.80
5	–	–	–	–	0.00	0.65

[a] Immunohistochemical scores indicate the intensity of staining of histologically distinct tissue within the same section. A dash (–) indicates that no tissue in the section exhibited the specific histopathologic feature. All samples were paraffin-embedded.
[b] Differentiating epithelial cells located in the superficial portion of intestinal mucosa.
[c] Epithelial cells deep within intestinal mucosa.
[d] Analysis included both adenomas and adenocarcinomas.

to detect GKLF by immunoblot analysis of extracts of human tumor cell lines that express the endogenous GKLF mRNA.

The cell type- and tumor type-specific patterns of GKLF mRNA expression were used to examine the specificity of αGKLF in immunohistochemical assays. These patterns can be summarized as follows. Human GKLF mRNA is detected by ISH in differentiating cells of oral epithelium, and is markedly elevated in oral tumors (17). The mRNA is not detected in morphologically normal basal or parabasal cells, particularly within epidermal pegs that extend further into the submucosa. Mouse GKLF mRNA is similarly found to be more highly expressed in superficial, differentiating cells of the skin and gut, and is reduced or absent in basal epithelial cells in both tissues (20, 23, 26). In contrast to human oral and breast cancer, GKLF mRNA expression is reduced in mouse colorectal tumors compared with normal epithelium (29), and is similarly reduced in human colorectal cancer as indicated by SAGE (31).

The staining pattern of αGKLF exhibited a strict concordance with detection of GKLF mRNA (Figs. 3 and 4, Table 2). In positive tissues, αGKLF exhibited a mixed nuclear and cytoplasmic staining pattern. For uninvolved epithelium, DCIS, and invasive breast carcinoma alike, the average cytoplasmic staining was 1.8- to 2.5-fold greater than nuclear staining, suggesting that subcellular localization was not altered during breast tumor progression in any consistent fashion. Cytoplasmic staining was subsequently used as a more sensitive indicator of overall expression.

In several samples of skin or oral squamous epithelium, αGKLF bound specifically to differentiating suprabasal epithelial cells (Fig. 3*A*). Compared with adjacent uninvolved epithelium, staining was markedly increased in malignant cells for each of several cases of squamous cell carcinoma with little or no staining of stromal components of the tumor, as shown previously for the mRNA (17). Likewise, staining was increased in superficial cells compared with cells deeper within epithelial crypts of the small bowel (Fig. 3*B*) or the large bowel (Table 2; $P = 0.043$). In contrast to oral and breast tumors, staining was reduced in tumor cells compared with adjacent superficial epithelial cells for each of four cases of human colorectal adenoma or carcinoma examined (Fig. 3*C*, Table 2; $P = 0.027$).

Expression of GKLF Protein Is Increased during Neoplastic Progression in the Breast. Eighteen cases were tested for GKLF expression by immunohistochemistry (Table 2, Fig. 4). Nuclear and cytoplasmic staining of normal breast epithelium, DCIS, and invasive carcinoma were semiquantitatively assessed. Low-level staining of tumor cells was observed for 6 cases (*e.g.*, cytoplasmic staining ranging from 0.20 to 0.85), with 11 cases exhibiting higher-level staining (*e.g.*, cytoplasmic staining ranging from 1.00 to 1.75). These results are consistent with detection of the mRNA in approximately two-thirds of tumors by ISH. For cases 23–31, which were analyzed by both ISH and immunohistochemical staining, results of the two methods exhibited a close correlation that reached statistical significance for invasive carcinoma cells ($N = 8$; coefficient = 0.77; $P = 0.024$). In DCIS, the correlation was moderate, although the sample number was small ($N = 7$; coefficient = 0.43). Perhaps because of the overall lower level of expression in uninvolved tissue, the correlation was weakest in uninvolved ducts. Minor differences observed for the two methods may be attributed to differences in sensitivity and specificity, to false negative results attributable to partial degradation of mRNA in some surgical samples, or to analysis of nonserial sections of the same tissue block. As observed in uninvolved tissue adjacent to

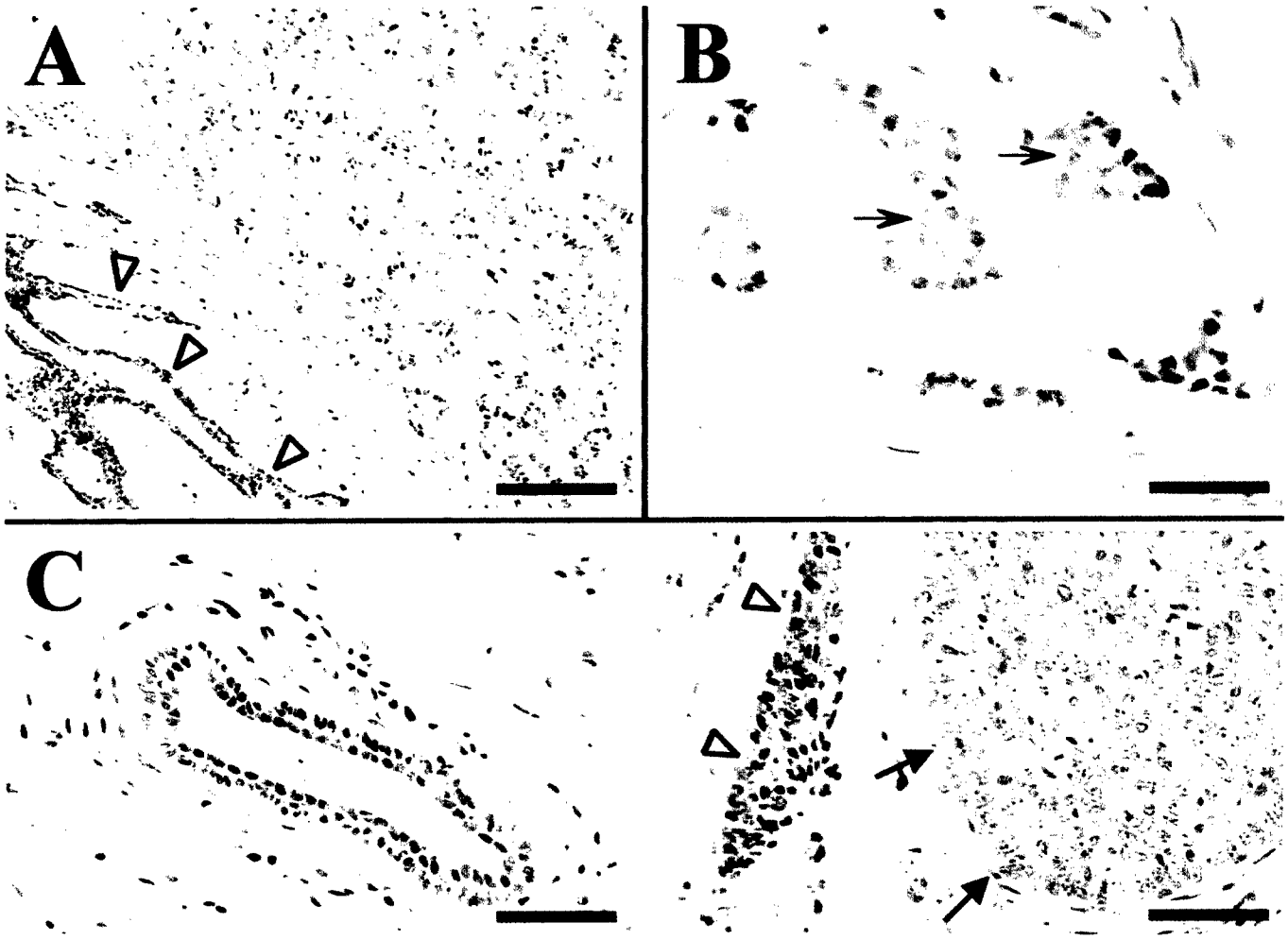

Fig. 4. Immunostaining of breast tissue with αGKLF. *A*, a tissue section containing uninvolved epithelium (*left, arrowheads*) adjacent to invasive carcinoma (*right*). *B*, a different case showing invasive carcinoma cells with a mixed nuclear and cytoplasmic staining pattern. *C*, a tissue section containing an uninvolved duct (*left panel*) adjacent to both DCIS (*right panel, arrows*) and invasive carcinoma (*right panel, arrowheads*). Scale bars: A = 120 μm; B = 30 μm; C = 60 μm.

tumors, staining was low or undetectable for each of five cases of reduction mammoplasty (data not shown).

Apparent GKLF expression as determined by nuclear or cytoplasmic immunostaining was increased in both DCIS and invasive carcinoma compared with uninvolved ducts (Table 2, Fig. 5). For morphologically normal ducts, staining of myoepithelial cells was not significantly different from that of luminal epithelial cells ($P = 0.303$, data not shown). However, staining of neoplastic cells in DCIS was significantly increased compared with myoepithelial cells within the same ducts ($P = 0.0001$), which was consistent with other studies indicating similarities between tumor cells and luminal epithelial cells (39).

Analysis of GKLF in Cultured Breast Epithelial Cells. Northern blot analysis of breast tumor cell lines revealed variable levels of GKLF expression relative to a tubulin control (Fig. 6). GKLF expression was high in MCF7 and ZR75-1; intermediate in BT474, BT20, MDAMB361, and SKBR3; and reduced in MDAMB453 and MDAMB231. Thus, expression in six of eight breast tumor-derived cell lines was increased relative to 184 cells, an HMEC population of finite life span derived from normal breast tissue after reduction mammoplasty (*Lane 1*). Expression was similarly increased in 184A1 cells (33). These immortalized cells were derived from 184 cells by treatment with benzo(*a*)pyrene. They are wild-type for p53 and $p105^{Rb}$ and are anchorage-dependent and nontumorigenic in animals. The results obtained for breast tumor cell lines support the conclusion that GKLF expression is up-regulated at the mRNA level in most breast tumors, whereas activation in 184A1 cells is consistent with identification of GKLF induction as an early event.

DISCUSSION

Oncogenes such as *c-MYC*, *GLI*, and *GKLF* function in a regulated fashion in normal epithelium to control cellular proliferation and differentiation (5, 6, 8, 23, 40, 41). Analysis of well-characterized tumor types such as colorectal carcinoma and basal cell carcinoma of the skin suggests that genetic alterations cluster within specific pathways, rather than within any specific gene, and that these pathways can function as regulators of oncogene transcription (42, 43). An activity common to several oncogenes implicated in carcinoma is the ability to induce transformed foci in the RK3E assay (17, 44, 45). This assay is highly specific, as foci result from expression of tumor-derived mutant (but not wild-type) alleles of *RAS* or *β-catenin* (Ref. 45 and data not shown), and only *GKLF* and *c-MYC* were identified in a large screen (17). The assay also detects a distinct subset of oncogenes compared with other host cell lines. With the exception of *RAS*, the oncogenes that transform RK3E cells do not induce foci in NIH3T3 cells (17, 44, 45).

GKLF encodes a zinc finger transcription factor of the GLI-Krüppel family (46) and is distinct from many other oncogenes in that expression in normal tissue is observed in terminally differentiating epithe-

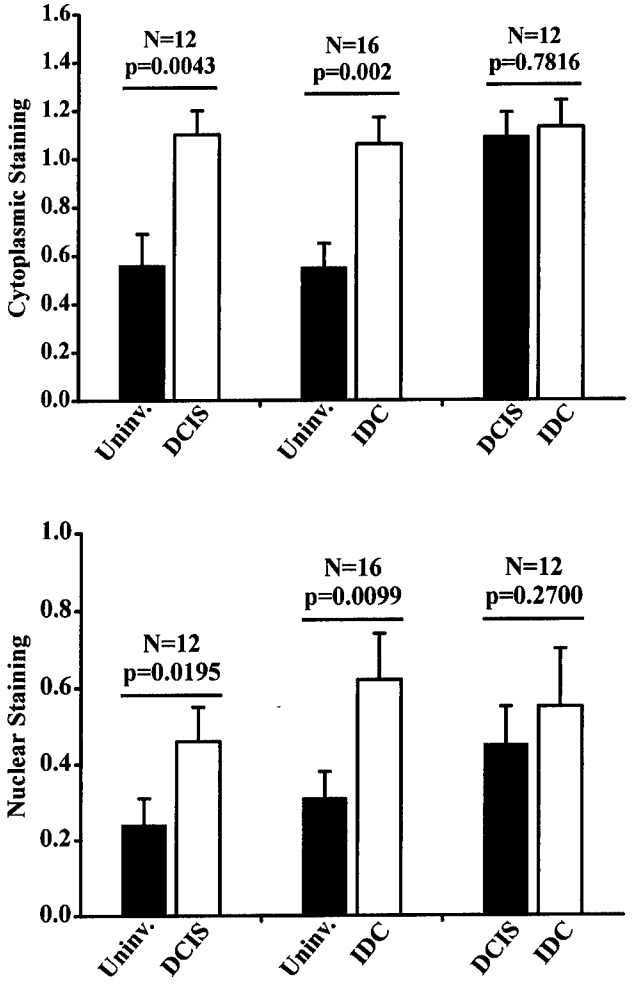

Fig. 5. Staining of uninvolved and neoplastic breast tissue by αGKLF. The data in Table 2 were analyzed using a paired *t* test. Sample size (*N*), statistical significance (*p*), and SE are indicated for each comparison. *Uninv.*, uninvolved ducts.

lial cells. In addition, expression is induced in association with cell growth-arrest *in vitro* (26). As predicted by these observations, expression in certain tumor-types is reduced compared with the relevant normal epithelia. Thus, GKLF expression is reduced in colorectal tumors, a result supported by multiple approaches including analysis of RNA extracted from tissues (29), SAGE (31), and immunohistochemical analysis of human tissues (this work). ISH analysis of several prostatic tumors likewise indicates that GKLF is expressed in normal prostatic epithelium, and that expression can be lost during tumor progression.

In contrast to colorectal and prostatic carcinoma, GKLF expression is activated in both invasive carcinoma and preinvasive neoplastic lesions during progression of most breast carcinomas and virtually all oropharyngeal squamous cell carcinomas. Breast and oral cancers share a number of additional molecular alterations. Loss-of-function mutations frequently affect p53 and p16/CDKN2, whereas a smaller proportion of tumors (5–20%) exhibit gene amplification of *c-MYC*, *cyclin D1*, erbB-family members including the EGF receptor and *erb*B-2/HER-2/*neu*, or others (47–51). Unlike carcinomas of the GI tract or skin, neither breast nor oral carcinoma is reported to exhibit frequent genetic alterations that activate known transforming oncogenes such as *RAS*, *β-catenin*, *c-MYC*, or *GLI*. By analogy with oncogenes in other tumor types, disruption of the pathways that control GKLF mRNA expression in breast epithelial cells and in oral mucosa represents a potential mechanism of tumor initiation or progression *in vivo*.

The pattern of GKLF expression in normal epithelia may provide clues as to how GKLF functions in tumor progression. Stratified squamous epithelium contains at least four functionally distinct compartments (52, 53). The stem cell compartment is composed of cells within the basal cell layer that exhibit a capacity for self-renewal, but which rarely divide. The transit-amplifying compartment is composed of cells within the basal or parabasal cell layers that exhibit rapid cell division but a reduced capacity for self-renewal. Differentiation occurs within the prickle cell layer that contains identifiable desmosomes, leading to the outermost, keratinized superficial layer. Whereas mechanisms regulating transitions from one compartment to the next remain poorly understood, c-MYC activation can induce stem cells to enter the highly proliferative transit-amplifying compartment (40). Because self-renewal and rapid cell division occur in distinct cell types, the organization of compartments enables the rapid turnover of epithelial cells while minimizing the possibility of sustaining permanent genetic damage in stem cells.

The observation that GKLF functions normally in the prickle cell layer suggests that each of the three compartments (stem cell, transit-amplifying, and prickle layer) expresses a transforming activity or a critical function (*e.g.*, self-renewal or proliferation) that may contribute to the progression of carcinoma. These compartments appear to be intermingled in dysplastic stratified squamous epithelium, with prickle layer markers including GKLF misexpressed in the basal layers, whereas other basal or parabasal markers are misexpressed in superficial layers. Loss of compartment-specific patterns of gene expression may result in coexpression of the properties of several compartments in a single cell. For example, specific properties of the prickle cell layer, such as reduced cellular adhesion to basement membranes, altered adhesion to other cells, and/or loss of the cellular mechanisms that mediate contact inhibition could confer invasive or metastatic properties to oral carcinomas.

To better understand the mechanism of transformation, we are characterizing transcriptional alterations induced by GKLF when expressed in epithelial cells *in vitro*. In the future, identification of upstream regulators of GKLF transcription in epithelial cells may elucidate the pathways that regulate GKLF and the mechanism of deregulation of GKLF in specific tumor-types.

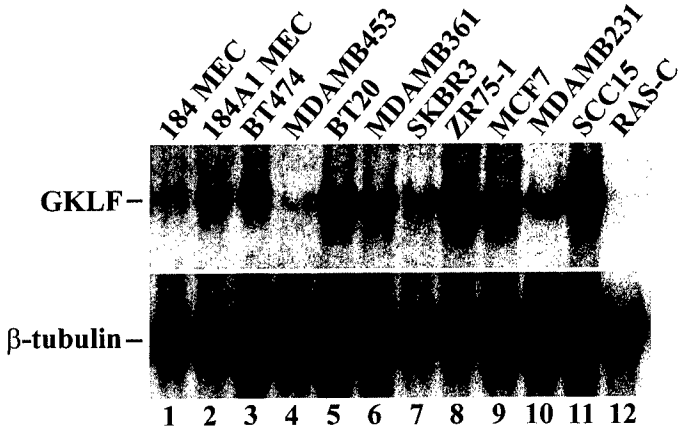

Fig. 6. Northern blot analysis of GKLF expression in human breast tumor cell lines. Total RNA from the indicated cell lines was analyzed. *Lane 1*, finite-life span HMECs. *Lane 2*, benzo(*a*)pyrene-treated, immortalized HMECs. *Lanes 3-10*, breast carcinoma-derived cell lines. *Lane 11*, SCC15, a human oral squamous cell carcinoma-derived cell line. *Lane 12*, a RAS-transformed rat cell line. The filter was stripped and hybridized to a β-tubulin probe.

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Oncogene Expression Cloning by Retroviral Transduction of Adenovirus E1A-immortalized Rat Kidney RK3E Cells: Transformation of a Host with Epithelial Features by c-MYC and the Zinc Finger Protein GKLF[1]

K. Wade Foster, Songrong Ren,[2] Iuri D. Louro, Susan M. Lobo-Ruppert, Peggy McKie-Bell, William Grizzle, Martha R. Hayes, Thomas R. Broker, Louise T. Chow, and J. Michael Ruppert[3]

Department of Biochemistry and Molecular Genetics [K. W. F., S. R., I. D. L., S. M. L-R., M. R. H., T. R. B., L. T. C., J. M. R.], Division of Hematology/Oncology, Department of Medicine [P. M-B., J. M. R.], Department of Pathology [W. G.], and Oral Cancer Research Center and Comprehensive Cancer Center [W. G., T. R. B., L. T. C., J. M. R.], University of Alabama at Birmingham, Birmingham, Alabama 35294-3300

Abstract

The function of several known oncogenes is restricted to specific host cells *in vitro*, suggesting that new genes may be identified by using alternate hosts. RK3E cells exhibit characteristics of epithelia and are susceptible to transformation by the G protein RAS and the zinc finger protein GLI. Expression cloning identified the major transforming activities in squamous cell carcinoma cell lines as c-MYC and the zinc finger protein gut-enriched Krüppel-like factor (GKLF)/epithelial zinc finger. In oral squamous epithelium, *GKLF* expression was detected in the upper, differentiating cell layers. In dysplastic epithelium, expression was prominently increased and was detected diffusely throughout the entire epithelium, indicating that *GKLF* is misexpressed in the basal compartment early during tumor progression. The results demonstrate transformation of epithelioid cells to be a sensitive and specific assay for oncogenes activated during tumorigenesis *in vivo*, and identify *GKLF* as an oncogene that may function as a regulator of proliferation or differentiation in epithelia.

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[2] Present address: Salk Institute for Biological Studies, La Jolla, CA 92037.

[3] To whom requests for reprints should be addressed, at Department of Medicine, Room 570 WTI, University of Alabama at Birmingham, Birmingham, AL 35294-3300. Phone: (205) 975-0556; Fax: (205) 934-9573; E-mail: mruppert@uab.edu.

Introduction

Cellular oncogenes have been isolated by characterization of transforming retroviruses from animal tumors, by examination of the breakpoints resulting from chromosomal translocation, by expression cloning of tumor DNA molecules using mesenchymal cells such as NIH3T3, and by other methods (1–5). Several human tumor-types exhibit loss-of-function mutations in a tumor suppressor gene that lead to activation of a specific oncogene in a large proportion of tumors. For example, *c-MYC* expression is regulated by the *APC* colorectal tumor suppressor; expression of *GLI* is activated by loss-of-function of *PATCHED1* in human basal cell carcinoma and in animal models; E2F is activated by loss-of-function of the retinoblastoma susceptibility protein $p105^{Rb}$; and RAS GTPase activity is regulated by the familial neurofibromatosis gene *NF1* (6–12). The comparative genomic hybridization assay and related methods have shown that numerous uncharacterized loci in tumors undergo gene amplification (13). These observations, and the infrequent genetic alteration of known oncogenes in certain tumor types, suggest that novel transforming oncogenes remain to be identified.

One limitation to the isolation of oncogenes has been the paucity of *in vitro* assays for functional expression cloning. Whereas most studies have used NIH3T3 or other mesenchymal cells as host for analysis of oncogenes relevant to carcinoma, the potential use of a host cell with epithelial characteristics has been discussed (2). In addition, several known oncogenes exhibit cell-type specificity. *GLI*, *BCR-ABL*, *NOTCH1/TAN1*, and the G protein GIP2 have been found to transform immortalized rat cells (14–18), but not NIHT3 cells, demonstrating the potential use of alternate assays for oncogene expression cloning.

A consistent feature of human tumors is inactivation of the G_1 phase cell cycle regulatory pathway that includes $p105^{Rb}$ (19–22). Loss-of-function mutations affect $p105^{Rb}$ or the cyclin-dependent kinase inhibitors, or gain-of-function mutations occur in cyclin-dependent kinases or associated cyclins. Such alterations are rate-limiting for tumor formation *in vivo* because inheritance of these defects predisposes to retinoblastoma, cutaneous malignant melanoma, and other tumors. During viral infection of normal cells, disruption of the same pathway is critical for successful induction of the cellular DNA replicative machinery to support viral replication. Therefore, viruses express proteins, such as adenovirus E1A, that affect cell cycle progression through direct interaction with cell cycle regulators including $p105^{Rb}$, $p27^{Kip1}$, and others (23–26).

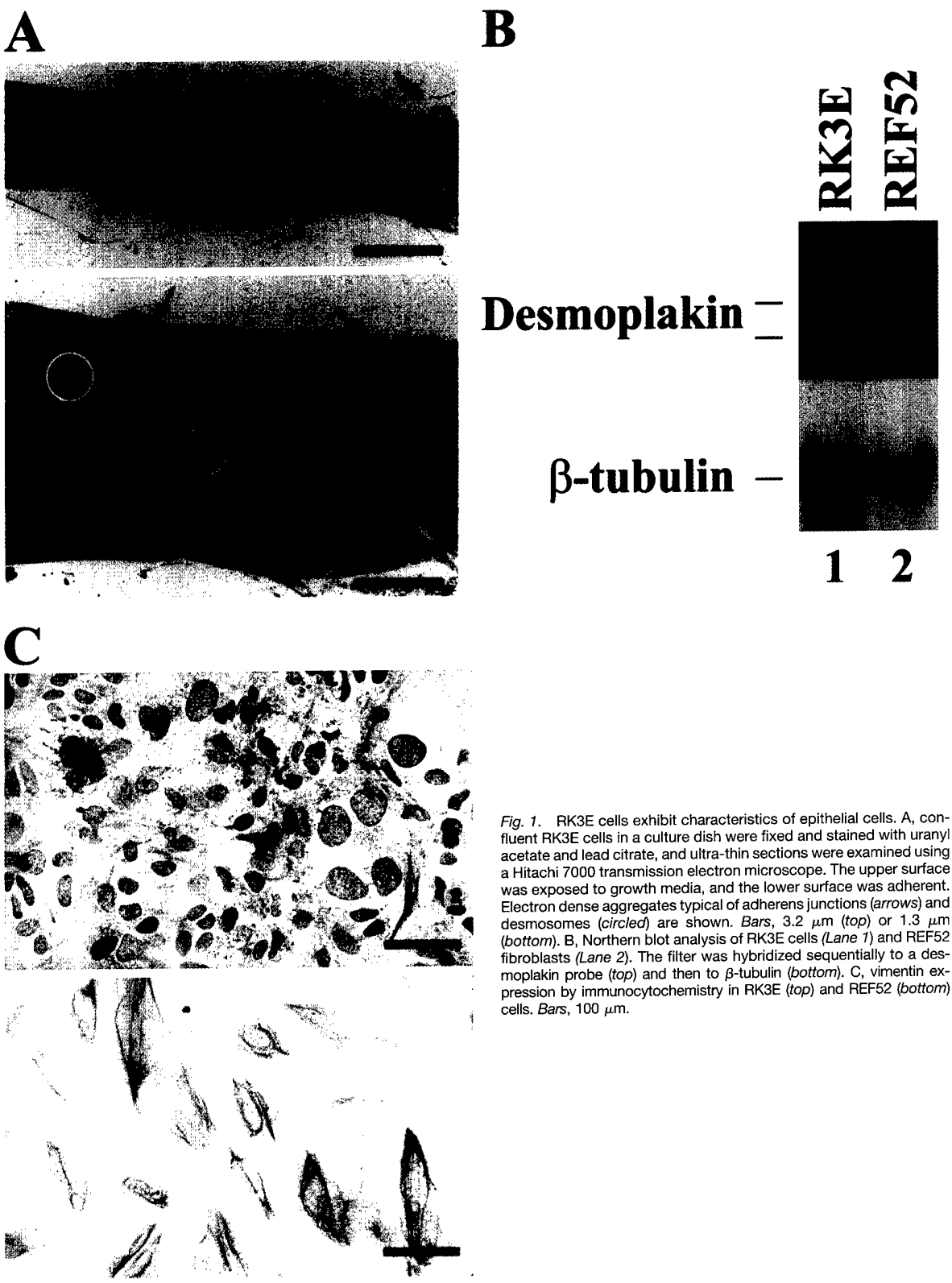

Fig. 1. RK3E cells exhibit characteristics of epithelial cells. A, confluent RK3E cells in a culture dish were fixed and stained with uranyl acetate and lead citrate, and ultra-thin sections were examined using a Hitachi 7000 transmission electron microscope. The upper surface was exposed to growth media, and the lower surface was adherent. Electron dense aggregates typical of adherens junctions (*arrows*) and desmosomes (*circled*) are shown. *Bars*, 3.2 μm (*top*) or 1.3 μm (*bottom*). B, Northern blot analysis of RK3E cells *(Lane 1)* and REF52 fibroblasts *(Lane 2)*. The filter was hybridized sequentially to a desmoplakin probe (*top*) and then to β-tubulin (*bottom*). C, vimentin expression by immunocytochemistry in RK3E (*top*) and REF52 (*bottom*) cells. *Bars*, 100 μm.

Table 1 Assessment of cDNA libraries

Library	λ titer	cDNA size (N,R)[a]	Probe[b]	cDNA clones transduced[c]	Transduced RK3E cells[d]	Foci identified
Squamous cell carcinoma	8.9×10^6	1.69 (10, 1.00–3.60)	NT	$\sim 4 \times 10^6$	$\sim 1.2 \times 10^7$	13
Breast carcinoma	7.4×10^6	1.64 (18, 0.50–2.7)	hBRF	$\sim 4 \times 10^6$	$\sim 1.2 \times 10^7$	1

[a] The mean size of cDNAs in kb pairs; the number of clones sized by gel electrophoresis (N) and the size range (R) are indicated.
[b] Plaques (420,000) were analyzed by hybridization to the 5′ end of the RNA polymerase III transcription factor hBRF cDNA (see "Results"); NT, not tested.
[c] The number of clones processed at each step of library construction was equal to or greater than 4×10^6. The *Bst* XI adapter strategy generates recombinant cDNA expression plasmids in an orientation-independent fashion, such that both sense and antisense vectors result.
[d] The number of RK3E cells transduced was estimated as the product of the transduction frequency (20%), the number of dishes screened (20), and the number of cells/dish (3×10^6).

We previously developed and used RK3E cells, immortalized by *E1A*, to demonstrate the transforming activity of *GLI* (17). We now show that these cells exhibit multiple features of epithelia and detect known and novel transforming activities in tumor cell lines. The epithelial features of the cells and/or the mechanism of immortalization may explain the surprising sensitivity and specificity of the assay compared with previous expression cloning approaches (27). Three of the four genes known to transform RK3E cells are activated by genetic alterations in carcinomas, and, of these genes, only *RAS* exhibits transforming activity in the commonly used host NIH3T3. We identify *GKLF*[4] (3) as an oncogene that is expressed in the differentiating compartment of epithelium and misexpressed in dysplastic epithelium. We also suggest that *GKLF* may regulate the rate of differentiation and maturation and the overall cellular transit time through epithelium. The functional similarities shared with other oncogenes, including *GLI* or *c-MYC*, identify *GKLF* as an attractive candidate gene relevant to tumor pathogenesis.

Results

RK3E Cells Have Characteristics of Epithelia. RK3E cells are a clone of primary rat kidney cells immortalized by transfection with adenovirus *E1A in vitro* (17). The cells exhibit morphological and molecular features that are epithelioid. They are contact-inhibited at confluence and are polarized with apical and basolateral surfaces and electron-dense intercellular junctions typical of adherens junctions and desmosomes (Fig. 1*A*). Northern blot analysis showed that RK3E cells, but not REF52 fibroblasts, expressed desmoplakin, a major component of desmosomes and an epithelial marker (Fig. 1*B*). By immunocytochemical staining, the mesenchymal marker vimentin was low or undetectable in RK3E cells, but was strongly positive in REF52 cells (Fig. 1*C*). Neither line reacted strongly with anticytokeratin or antidesmin antibodies. These results are consistent with the observation that E1A induces multiple epithelial characteristics without inducing cytokeratin expression (28).

Karyotype analysis revealed RK3E cells to be diploid with a slightly elongated chromosome 5q as the only apparent abnormality (17). Importantly, RK3E cells can be transformed by functionally diverse oncogenes such as *RAS* and *GLI*. Four such transformed lines were each homogeneous for DNA content, as determined by fluorescence analysis of propidium iodide-stained cells derived from *RAS*- (one line) or *GLI*- (three lines) induced foci, indicative of a relatively stable genetic constitution (data not shown). These properties suggested that RK3E cells may serve as an *in vitro* model for identification and mechanistic analysis of gene products involved in the progression from normal epithelial tissue to malignancy.

cDNA Library Construction. To identify transforming genes, we used mRNA from human squamous cell carcinoma- or breast tumor-derived cell lines. These tumor types do not exhibit frequent alteration of *RAS* or *GLI*. After pooling mRNAs for each tumor type, oligo dT-primed cDNA libraries were constructed in bacteriophage λ (Table 1). The libraries were high-titer (assessed before amplification on agar plates) with a mean insert size of 1.6–1.7 kb. The amplified breast cDNA library was further assessed by plaque screening for the transcription factor hBRF, using a probe derived from the 5′ end of the protein coding region (bases 315–655, accession U75276). Each of the seven clones identified were derived from independent reverse transcripts, as determined by end sequencing, confirming that complexity of the library was maintained during amplification. The inserts ranged in size from 2.1–3.4 kb and contained the entire 3′ UTR and much or all of the protein coding region intact. Three of the seven clones extended through the predicted initiator methionine codon, whereas four others were truncated further downstream. These results suggested that the library is relatively free of COOH-terminally truncated clones and contains full-length cDNAs even for relatively long mRNAs. The overall abundance of hBRF mRNA has not been determined.

Isolation of c-MYC and GKLF by Expression Cloning. The libraries were cloned into the MMLV retroviral expression plasmid pCTV1B (27), packaged in BOSC23 cells (29), and high-titer virus supernatants were applied to RK3E cells. Fourteen foci, identified at 10–20 days after transduction, were individually expanded into cell lines. Thirteen of these foci contained a single stably integrated cDNA, as indicated by PCR (Fig. 2*A*). Eleven of these PCR products were identified as human *c-MYC* by end-sequencing and restriction enzyme analysis. The *c-MYC* cDNA in *Lane 15* included the coding region and 193 bases of 5′ UTR sequence (accession V00568). As determined by sequencing or restriction mapping, the other *c-MYC* cDNAs extended further 5′ *(Lanes 1, 3, 5–7, 9–7, 13,* and *14)*, such that all of the clones contained the entire protein-coding region.

[4] The abbreviations used are: GKLF, gut-enriched Krüppel-like factor; β-gal, β-galactosidase; UTR, untranslated region; MMLV, Moloney murine leukemia virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ISH, *in situ* hybridization.

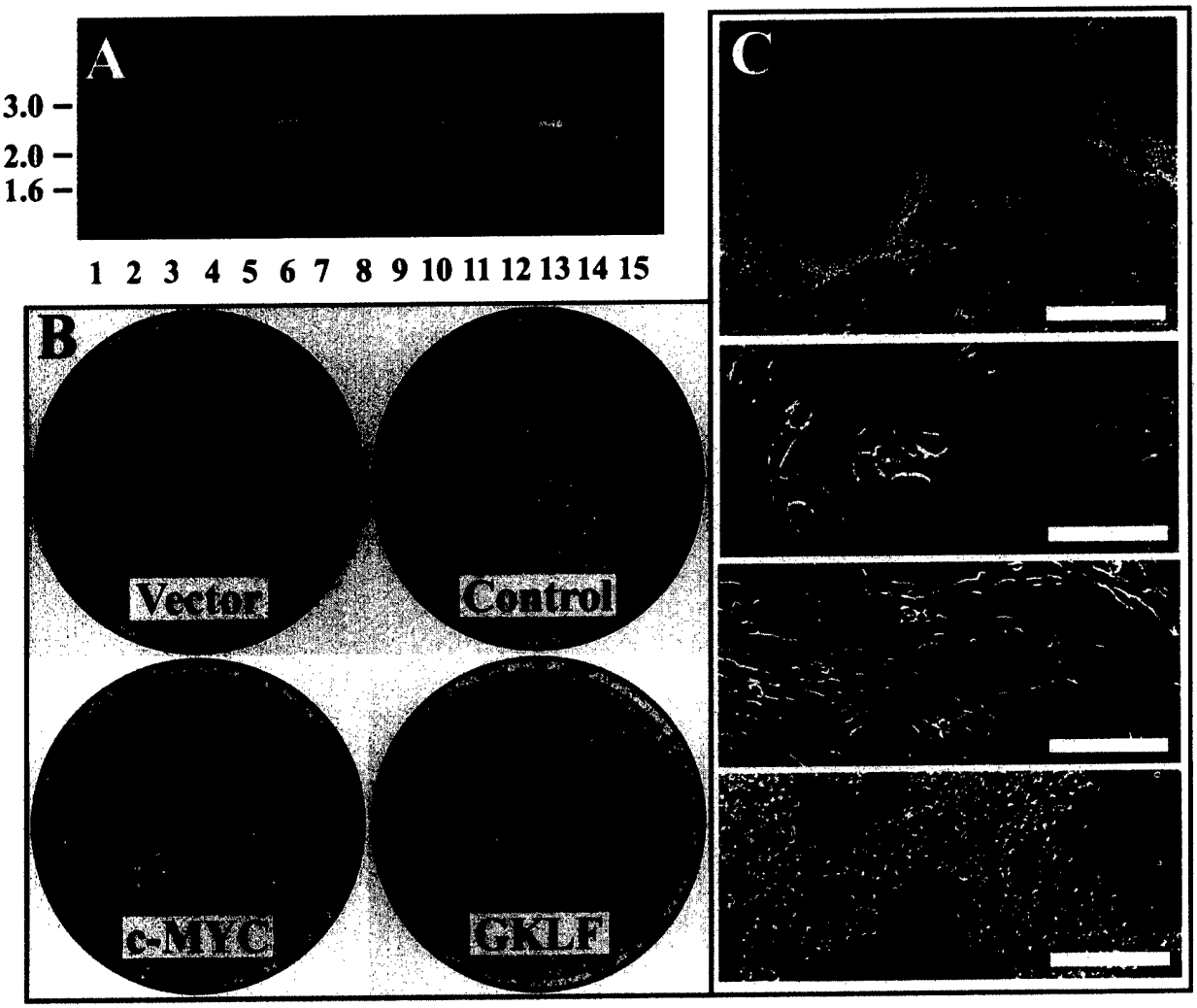

Fig. 2. Expression cloning of *c-MYC* and *GKLF*. A, identification of human cDNAs present in transformed RK3E cell lines SQC1-SQC13 (derived using a squamous cell carcinoma library, *Lanes 1* and *3–14*) and BR1 (derived using a breast carcinoma library, *Lane 15*). PCR was used in combination with vector-derived primers and cell line genomic DNA. RK3E genomic DNA served as a negative control template *(Lane 2)*. No cDNA was retrieved from cell line SQC3 *(Lane 4)*. All foci identified in the screen are represented. Molecular weight markers are indicated on the left in kb pairs. B, reconstitution of transforming activity by cloned PCR products. cDNAs were cloned into a retroviral expression plasmid, packaged into virus using BOSC23 cells, and applied to RK3E cells. Foci were fixed and stained at 3–4 weeks. *Vector*, pCTV3K; *Control*, pCTV3K-SQC1; *c-MYC*, pCTV3K-BR1; *GKLF*, pCTV3K-SQC7. C, morphology of foci and cloned cell lines. *Top to bottom*: *first panel*, low-power phase contrast view of adjacent foci in a dish transduced with retrovirus encoding *GKLF* (*bar*, 900 μm); *second through fourth panels*, high-power phase contrast view (*bar*, 230 μm); *second panel*, RK3E cells at subconfluence; *third panel*, *GKLF*-transformed RK3E cells; *fourth panel*, *c-MYC*-transformed RK3E cells.

In addition, two cell lines (Fig. 2*A*, *Lanes 8* and *12*) contained cDNAs coding for *GKLF*. Mouse and human *GKLF* cDNAs were previously isolated by hybridization with zinc finger consensus probes (30–32), but were not implicated as oncogenes or found to be induced during neoplastic progression. After cloning into plasmid, the sequences of these two cDNAs, termed SQC7 and SQC11, were obtained in total. As determined by comparison with multiple expressed sequence tags and two full-length coding sequence files in the database (accessions U70663 and AFO22184), each contained the predicted GKLF protein coding region bounded by 5′ and 3′ UTRs. An ATG in good context for translation initiation was located at base 330, with the predicted terminator codon at base 1740. Both isolates were artificially truncated at the *Xho*I site in the 5′ UTR during library preparation. Because the transcripts had been processed using distinct AAUAAA polyadenylation signals, the cDNAs were slightly different in length and derived from independent mRNA molecules (Fig. 2*A*).

Sequencing revealed these two *GKLF* isolates to be identical within the residual 5′ UTR and throughout the coding region. A single bp difference in the 3′ UTR represents a PCR-induced error or a rare variant, as determined by comparison with ESTs. Comparison to a placenta-derived sequence (accession U70663) revealed three single bp differences in the coding region. These differences were resolved by alignment with other sequences in the database (accessions AF022184 and AA382289) from normal tissues, indi-

Table 2 Retroviral transduction of reconstituted *GKLF* and *c-MYC* expression vectors

Plasmid	Focus assay (no. foci/10 cm dish)[a]	Colony morphology assay (no. transformed/total)[b]
pCTV3K (vector)	0, 0	0/184
pCTV3K-SQC1[c] (*c-MYC*)	0, 0	0/232
pCTV3K-SQC5 (*c-MYC*)	>1000, >1000	ND[d]
pCTV3K-BR1 (*c-MYC*)	>1000, >1000	81/91 (89%)
pCTV3K-SQC7 (*GKLF*)	>1000, >1000	91/206 (44%)
pCTV3K-SQC11-2[e] (*GKLF*)	>1000, >1000	ND
pCTV3K-SQC11-3 (*GKLF*)	>1000, >1000	ND

[a] RK3E cells were transduced with 4 ml of virus supernatant after calcium phosphate-mediated plasmid transfection of virus packaging cells.
[b] RK3E cells were transduced with 0.4 ml of thawed viral supernatant. Cells were split 1:4 into selective media 30 h later. At 2 weeks, drug-resistant colonies were fixed, stained, and examined visually for morphological transformation. Numbers indicate colonies/10-cm dish. A duplicate transduction experiment yielded similar results (data not shown). No colonies formed in control dishes that were not exposed to virus.
[c] pCTV3K-SQC1 is a *c-MYC* allele obtained by PCR that exhibited greatly reduced transforming activity compared with other alleles.
[d] ND, not determined.
[e] SQC11-2 and -3 are independent plasmid clones derived from the same PCR reaction (Fig 2*A*, *Lane 12*).

cating that the *GKLF* molecules obtained by expression cloning are predicted to encode the wild-type protein.

Reconstitution of Transforming Activity for *c-MYC* and *GKLF*. To demonstrate transforming activity, three independent PCR products each for the *c-MYC* and *GKLF* cDNAs were cloned into the retroviral expression vector pCTV3K (27), packaged into virions, and tested for transformation of RK3E cells *in vitro* (Fig. 2, *B* and *C*; Table 2). One of the *c-MYC* clones (pCTV3K-SQC1) possessed greatly reduced transforming activity in multiple experiments despite similar viral titers, as determined by induction of hygromycin resistance, suggesting that an error may have been introduced during PCR. Each of the other virus supernatants carrying *GKLF* and *c-MYC* transgenes induced >1000 foci/dish compared with no foci for virus controls.

To determine the efficiency of transformation by *GKLF* and *c-MYC*, a colony morphology assay was used, as described previously (27). Virally transduced cells were selected in hygromycin at low confluence, and stable colonies were fixed, stained, and scored for morphological transformation by visual inspection as above for foci (Table 2). The *c-MYC*-transduced cells exhibited loss of contact inhibition and dense growth in 89% of colonies. The *GKLF*-transduced cells exhibited a transformed morphology in 44% of colonies. In comparison, a previous study showed that 70% and 40% of NIH3T3 colonies transduced by viruses carrying *RAS* and *RAF* exhibited a transformed morphology (27). We, likewise, tested virus supernatants for transformation of NIH3T3 cells. Neither *c-MYC* nor *GKLF* induced morphological transformation of NIH3T3 colonies, as previously described for *GLI* and others (data not shown; Ref. 17). These results identify the RK3E assay as not only highly specific, but also sensitive to the activity of a select group of oncogenes.

In lieu of sequencing the *c-MYC* alleles, we confirmed that wild-type *c-MYC* can transform RK3E cells. A human wild-type expression vector (pSRαMSV *c-MYC* tk-neo) induced foci using direct plasmid transfection of RK3E cells in multiple experiments. Foci were observed at a similar frequency using known wild-type or new *c-MYC* isolates when analyzed in parallel (data not shown). In addition, retrovirus encoding the estrogen receptor-c-MYC (wild-type) fusion protein induced morphological transformation of RK3E cells in the presence or absence of 4-hydroxy-tamoxifen (33). No effect was observed for controls (empty vector or a control containing a deletion in c-MYC residues 106–143).

Northern blot analysis of transformed RK3E cell lines demonstrated expression of the *c-MYC* and *GKLF* vector-derived transcripts (Fig. 3*A*). No endogenous transcripts were detected at the stringency used in this experiment. Compared with RK3E cells at subconfluence *(Lane 1)* or confluence *(Lane 2)*, no consistent increase of *E1A* transcripts was detected in cells transformed by *RAS*, *GLI*, *c-MYC*, or *GKLF*, suggesting that these genes act upon cellular targets to induce transformation.

To detect the endogenous rat *GKLF* transcript, we used reduced-stringency wash conditions and a *Sma*I fragment from the coding region exclusive of the COOH-terminal zinc fingers and with no sequence similarity to other genes in the database. By this approach, the apparent *GKLF* transcript was identified and migrated at 3.1 kb, similar to the human 3.0-kb transcript, in RK3E and all derivative-transformed cell lines (data not shown). A single transcript with the same mobility was detected by hybridization of the filter to full-length coding region probe. These studies revealed similar *GKLF* expression in RK3E and in derivatives transformed by *RAS*, *GLI*, or *c-MYC*. The results show that *GKLF* mRNA expression is not significantly altered by these other oncogenes and is consistent with function of *GKLF* in an independent pathway.

Cell lines derived from foci induced by *c-MYC* or *GKLF* were further tested for tumorigenicity in athymic mice by s.c. inoculation at four sites for each line (Table 3; Ref. 17). Tumors were >1 cm in diameter and were scored at 2–4 weeks after inoculation. Cells transformed by *c-MYC* induced tumors in 75% or 100% of sites injected (two lines tested). Three lines transformed by *GKLF* each induced tumors in 50–75% of sites injected. No tumors resulted from injection of RK3E cells, whereas a *GLI*-transformed cell line induced tumors in each of the four sites injected. In all, *GKLF* cell lines induced tumors in 8 of 12 injection sites, compared with 7 of 8 injection sites for *c-MYC* and 4 of 4 injection sites for *GLI*. *GKLF*-induced tumors also grew more slowly *in vivo*, reaching 1 cm in diameter by 3.4 weeks, on average, compared with 2.6 weeks for c-MYC and 3 weeks for *GLI*. The

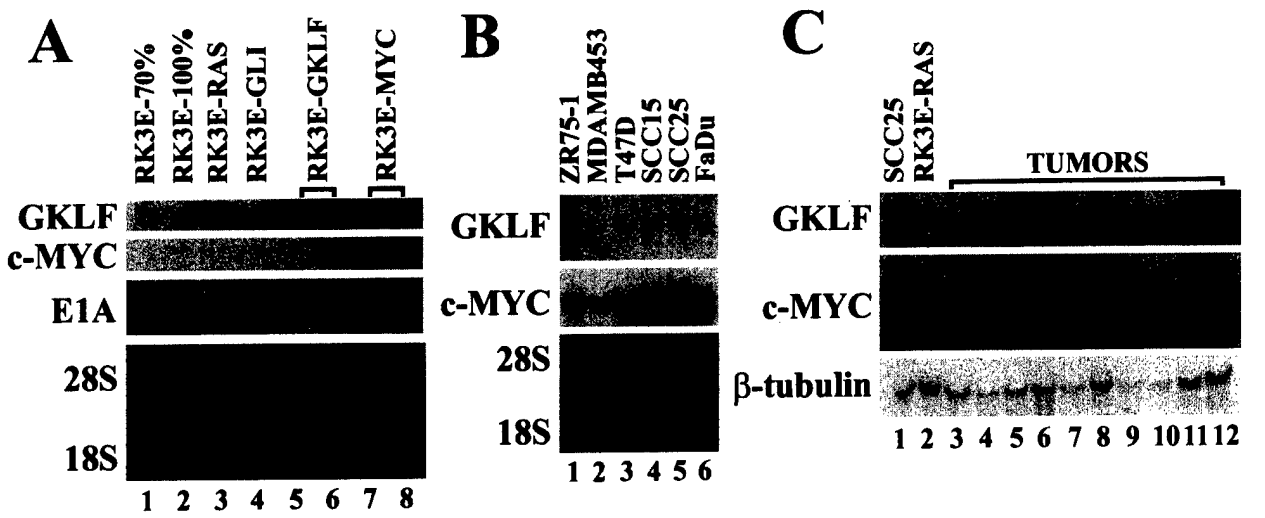

Fig. 3. Northern blot analysis of *c-MYC* and *GKLF* expression. Total RNA (25 μg) was loaded for each sample. *A*, analysis of transgene expression in RK3E cells and derivative cell lines transformed by the indicated oncogene. *Lane 1*, RK3E cells in exponential growth phase; *Lane 2*, RK3E cells incubated at confluence for 5 days. Ethidium bromide-stained RNA is shown below after transfer to the filter. *B*, endogenous *GKLF* (3.0 kb) or *c-MYC* (2.3 kb) expression in tumor cell lines. *Lanes 1–3*, breast cancer lines; *Lanes 4–6*, squamous cell carcinoma lines. *C*, analysis of gene expression in laryngeal squamous cell carcinoma. *Lane 1*, SCC25 cell line; *Lanes 3–6*, *9*, and *12*, primary tumors; *Lanes 7*, *8*, *10*, and *11*, metastatic tumors; *Lanes 3–12* correspond to case numbers 5, 8, 18–20, 6, and 21–24, respectively (see Table 4). RK3E-RAS cell RNA served as a negative control *(Lane 2)*, whereas hybridization to β-tubulin served as a control for loading.

Table 3 Tumorigenicity of RK3E-derived cell lines in athymic mice

Cell line	No. tumors/ no. sites injected	Tumor latency *in vivo* (weeks)[a]	Doubling time *in vitro* (h)
RK3E	0/4	–	12.7
RK3E-c-MYC BR1[b]	3/4	3,3,4	19.1
RK3E-c-MYC B[c]	4/4	2,2,2,2	19.8
RK3E-GKLF E	3/4	3,3,3	33.7
RK3E-GKLF F	2/4	4,4	27.0
RK3E-GKLF G	3/4	3,3,4	ND[d]
RK3E-GLI	4/4	3,3,3,3	18.0

[a] The time required for tumors to reach 1 cm in diameter is indicated.
[b] Cell line derived from a focus identified in the original screen using a breast cancer cDNA library.
[c] Cell line derived by transformation with the reconstituted plasmid pCTV3K-BR1.
[d] ND, not determined.

moderately increased latency and decreased efficiency of tumor formation for *GKLF* cell lines may be attributable to the intrinsic rate of proliferation for these cells (Table 3). Although *c-MYC*, *GLI*, and *GKLF* cell lines all exhibited prolonged doubling times *in vitro* compared with RK3E cells, *GKLF* cells divided more slowly than the other transformed cell lines.

Northern Blot Analysis of Tumors and Tumor-derived Cell Lines. We then examined human tumors and cell lines by Northern blot analysis of total RNA (Fig. 3, *B* and *C*). *GKLF* expression in breast or squamous cell carcinoma cell lines was variable, with increased expression in the breast tumor line ZR75–1 and the squamous cell lines SCC15 and SCC25 (Fig. 3*B*). In human squamous cell carcinomas microdissected to enrich for tumor cells, *GKLF* expression was detected in each of 10 primary or metastatic tumors analyzed, with expression levels comparable with that for the cell line SCC25 (Fig. 3*C*). The results suggest that *GKLF* represents a potent transforming activity that is consistently expressed in tumors as well as in tumor-derived cell lines. Because *GKLF* was isolated from cell lines that express the gene at a level found in tumors *in vivo*, the results suggest that GKLF may represent a major transforming activity in tumors, as well as in cell lines.

Gene Copy Number of *c-MYC* and *GKLF*. c-MYC was previously shown to be activated by gene amplification in ~10% of oral squamous cancers and may be activated in these or other tumors by genetic alteration of WNT-*APC*-β-catenin pathway components (6, 34–37). To determine whether expression of *GKLF* in cell lines and tumors is, likewise, associated with gene amplification, we performed Southern blot analysis (Fig. 4, *A* and *B*). Filters were sequentially hybridized to *GKLF*, *c-MYC*, and β-tubulin. Increased copies of *c-MYC* were identified in two cell lines used for library construction, FaDu and MCF7. Increased hybridization to *c-MYC* was, likewise, observed for 1 of 11 oral squamous cell carcinomas (Fig. 4*A*, *Lane 10*) and for one of nine breast carcinomas (Fig. 4*B*, *Lane 8*). These results are consistent with the published frequencies of *c-MYC* amplification for these tumor types (34, 35, 38). No copy number gains of *GKLF* were observed, indicating that other mechanisms may contribute to expression of GKLF in tumors. The same may be true for *c-MYC* because gene amplification in FaDu cells was associated with reduced expression compared with other oral cancer cell lines (Fig. 3*B*).

***GKLF* Expression Is Activated Early during Tumor Progression *in Vivo*.** Previously, expression of c-MYC was found to be up-regulated consistently in dysplastic oral mucosa and in squamous cell carcinomas, and tumors with the highest levels of c-MYC expression were associated with the

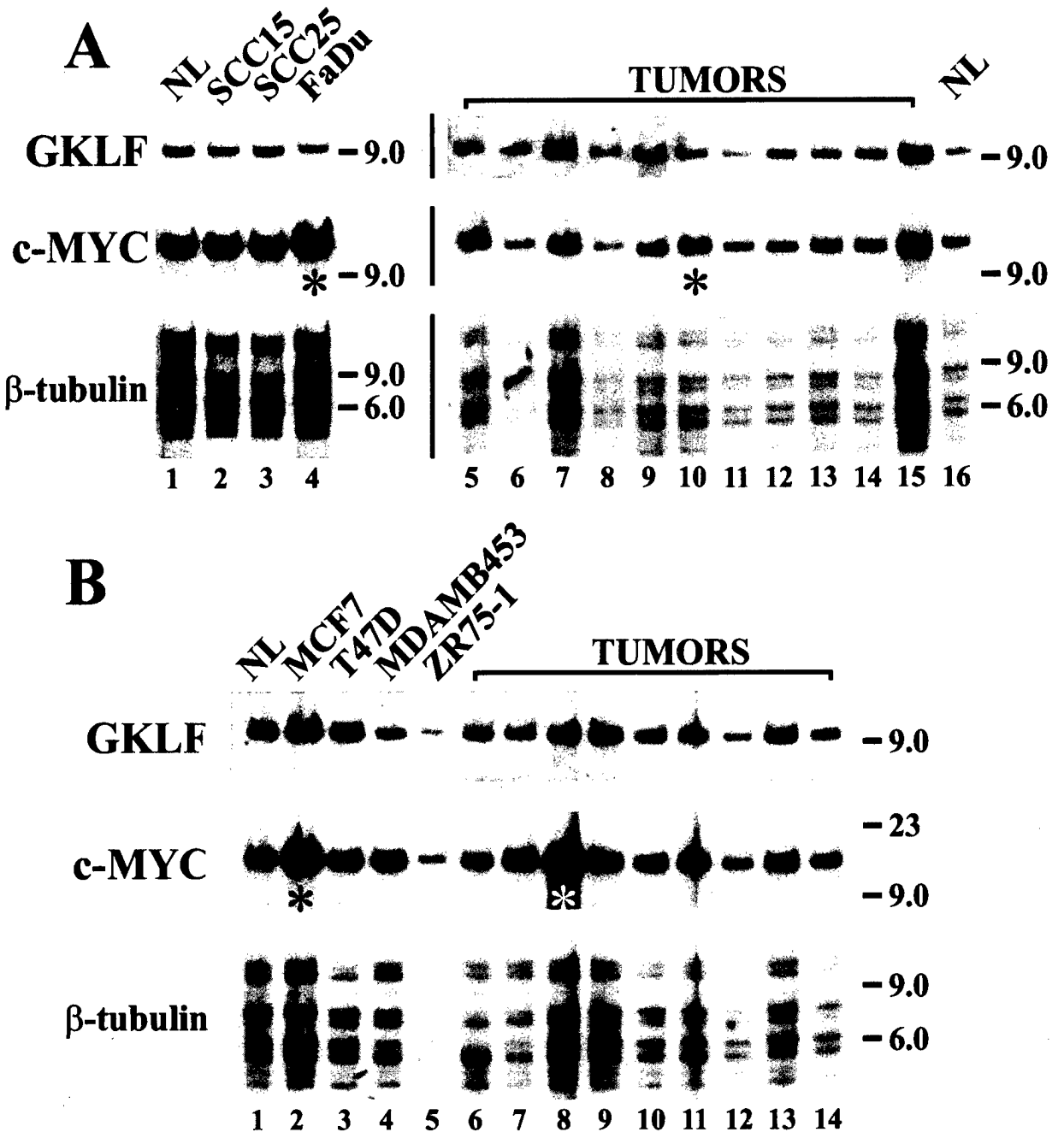

Fig. 4. Southern blot analysis of cell line- and tumor-derived genomic DNA. DNA (5 μg) was digested with *Eco*RI and separated by gel electrophoresis. The filters were hybridized sequentially to GKLF, c-MYC, and β-tubulin probes. *, samples with increased apparent copy number of c-MYC. Molecular weight markers are indicated on the *right*. *NL*, normal human lymphocyte DNA. *A*, oropharyngeal squamous cell carcinoma. Cell lines *(Lanes 2–4)* and tumors *(Lanes 5–15)* are shown. *B*, breast carcinoma. Cell lines *(Lanes* 2–5) and tumors *(Lanes 6–14)* are shown.

poorest clinical outcome (36, 39–41). To determine how *GKLF* mRNA expression is altered during tumor progression, we analyzed squamous cell carcinoma of the larynx and adjacent uninvolved epithelium from the same tissue blocks using ^{35}S-labeled riboprobes by ISH analysis. In apparently normal epithelium, *GKLF* expression was detected in the spinous layer above the basal and parabasal cells (nine specimens analyzed; Fig. 5, *A-C*, *G-I*; Table 4). No specific *GKLF* expression was detected in the basal or parabasal cells or in the underlying dermis. In contrast, a sense control probe produced grains at a much-reduced frequency in a uniform fashion across the epithelium. *GAPDH* expression served as a positive control and was detected diffusely throughout the entire epithelium (data not shown). The ob-

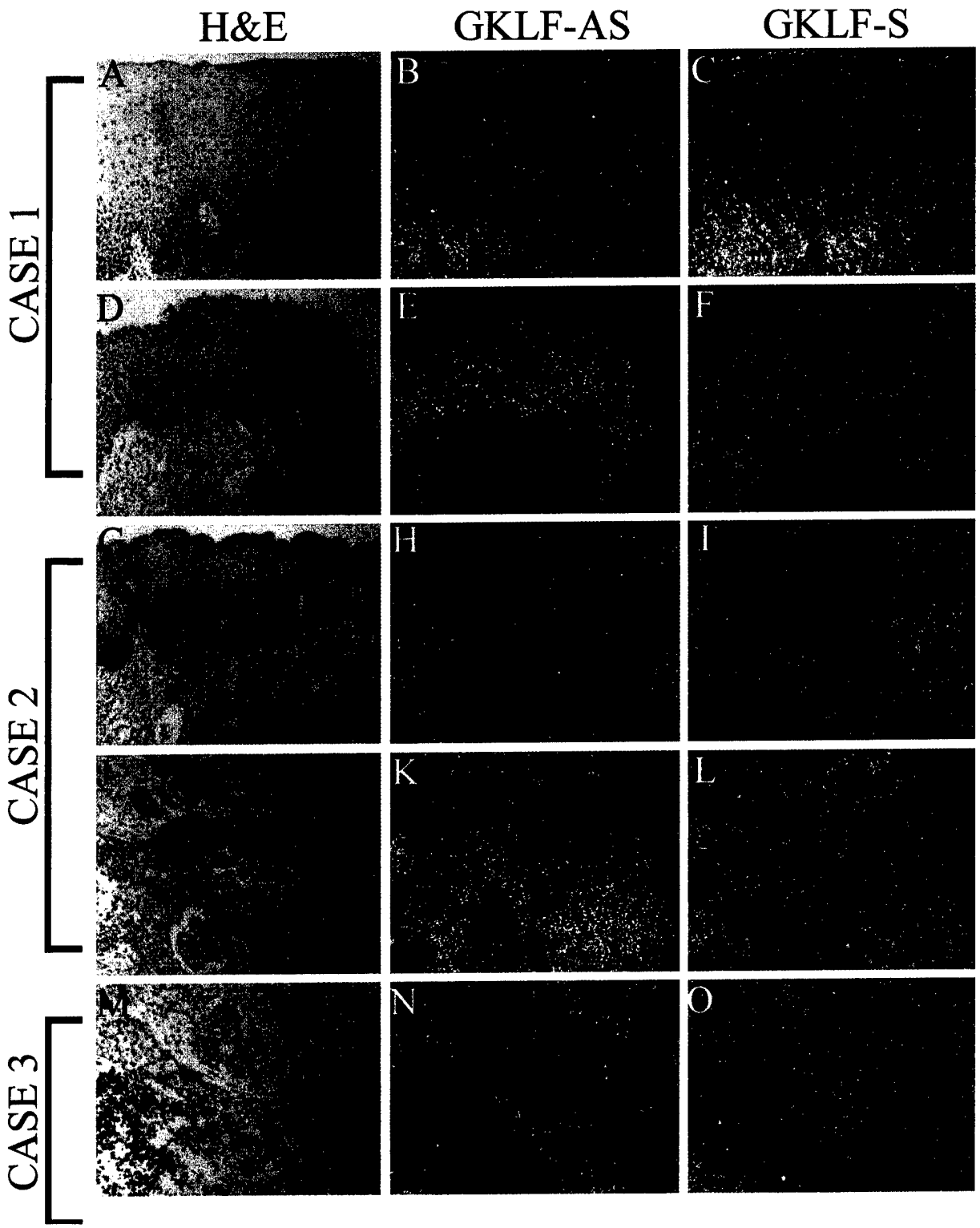

Fig. 5. ISH analysis of *GKLF*. Paraffin-embedded (*A-L*) or fresh-frozen (*M-O*) tissues were analyzed using antisense (*GKLF-AS*) or sense (*GKLF-S*) ^{35}S-labeled RNA probes. Each image (*A-O*) is 650 μm × 530 μm. Sections were stained with H&E. *Case 1*, *A-C*, uninvolved epithelium in a patient with primary laryngeal squamous cell carcinoma; *D-F*, adjacent dysplastic epithelium within the same tissue block. *Case 2*, *G-I*, uninvolved epithelium; *J-L*, adjacent primary tumor nests within stroma in the same tissue block; *, a salivary gland and ducts. *Case 3*, *M-O*, metastatic laryngeal squamous cell carcinoma infiltrating a lymph node; *, lymphocytes.

Table 4 Expression of *GKLF* in oral epithelium and tumors

Case[a]	Histopathology (U,[b]D,P,M)	Tissue source (PE/FF)	Method (N/ISH)	GKLF expression
1	U,D,P	PE	ISH	D,P>U
2	U,D	PE	ISH	D>U
2	U,P	PE	ISH	P>U
3	M	FF	ISH	+
4	U,D	PE	ISH	D>U
5	P	FF	N,ISH	+
6	M	FF	N,ISH	+
7	P	FF	ISH	+
8	P	FF	N,ISH	+
9	D,P	PE	ISH	D,P+
10	M	PE	ISH	+
11	U,D,P	PE	ISH	D,P>U
12	U,D	PE	ISH	D>U
12	U,D,P	PE	ISH	D,P>U
13	U	PE	ISH	+
13	P	PE	ISH	+
14	P	PE	ISH	+
14	M	PE	ISH	+
15	D	PE	ISH	+
15	D	PE	ISH	+
15	D,P	PE	ISH	D,P+
16	U,D,P	PE	ISH	D,P>U
16	M	PE	ISH	+
17	D,P	PE	ISH	D,P+
18	P	FF	N	+
19	P	FF	N	+
20	M	FF	N	+
21	P	FF	N	+
22	M	FF	N	+
23	M	FF	N	+
24	P	FF	N	+

[a] Each row corresponds to a tissue specimen. Levels of gene expression indicate changes identified within, rather than between, single tissue sections. For some cases, multiple specimens isolated during the same surgical procedure were analyzed. ISH results were confirmed by analysis of sections in duplicate.

[b] U, uninvolved or normal-appearing epithelium; D, dysplastic epithelium; P, primary tumor; M, metastatic tumor; PE, paraffin-embedded; FF, fresh-frozen; N, Northern; D,P>U, increased expression in dysplasia and primary tumor compared with uninvolved epithelium in the same section; D,P+, expression in both dysplasia and adjacent primary tumor.

served pattern of *GKLF* expression is identical to the pattern in normal mouse skin (32).

For each of 12 specimens analyzed, dysplastic epithelium exhibited increased *GKLF* expression throughout the epithelium (Fig. 5, *D-F*; Table 4, *Cases 1, 2, 4, 9, 11, 12,* and *15–17*). In contrast to results obtained in normal-appearing epithelium, there was no reduction of expression in the basal and parabasal layers compared with superficial layers. For tissue sections that contained both uninvolved epithelium and adjacent dysplastic epithelium, the overall level of *GKLF* expression in dysplastic epithelium was prominently elevated compared with the *GKLF*-positive cell layers in uninvolved epithelium (Fig. 5, *B*, *E*, and *H*; Table 4, *Cases 1, 2, 4, 11, 12,* and *16*). These results suggest that *GKLF* expression is qualitatively and quantitatively altered in dysplasia, that exclusion of *GKLF* from the basal and parabasal cell layers is lost early during neoplastic progression, and that *GKLF* exhibits properties of an oncogene not only *in vitro*, but also *in vivo*.

As shown by Northern blot analysis, *GKLF* transcripts are consistently present in tumor-derived mRNA (Fig. 3*C*; Table 4). To determine whether *GKLF* is expressed in tumor cells, we examined laryngeal squamous cell carcinomas by mRNA ISH. Expression was detected in each primary (13 cases) or metastatic (5 cases) tumor examined (Fig. 5, *J-O*; Table 4), with all or nearly all tumor cells associated with silver grains. The level of expression was somewhat heterogeneous, with higher levels found in the periphery and in nodules of tumor containing centrally necrotic cells or keratin pearls. As for dysplastic epithelium, expression in tumor cells was consistently elevated compared with uninvolved epithelium in the same sections (Fig. 5, *H* and *K*; Table 4, *Cases 1, 2, 11, 12,* and *16*). However, expression in tumor cells was not higher than in dysplastic epithelium (*Cases 1, 9, 11, 12,* and *15–17*). For several cases, expression in the most dysplastic epithelium was higher than in adjacent *GKLF*-positive tumor, suggesting that *GKLF* expression is specifically activated during the transition from normal epithelium to dysplasia, before invasion or metastasis.

Discussion

The results demonstrate that cells with an epithelial phenotype can be used for identification of transforming activities present in carcinoma-derived cell lines. The assay repeatedly identified two genes, and none of the isolated cDNAs were artificially truncated or rearranged within the protein coding region. This indicates that transformation of these cells is unusually specific to a few pathways or genes, including *c-MYC*, *GKLF*, *RAS*, and *GLI*. *c-MYC*, *RAS*, and *GLI* are directly or indirectly activated by genetic alterations in diverse carcinoma types during tumor progression *in vivo* (9, 10, 42–44). For both breast and oral squamous carcinoma, the tumor types analyzed in this study, *c-MYC* gene amplification is one of the more frequent oncogene genetic alterations and is observed in 10–15% of cases. By analogy, novel oncogenes identified by the RK3E assay may be directly activated in neoplasms through gain-of-function mutations or indirectly activated by loss-of-function genetic alterations.

Whitehead *et al.* (27) developed the retroviral vectors that we used in this study for transduction of NIH3T3 cells, in which they isolated 19 different cDNAs encoding 14 different proteins. Known oncogenes were isolated, including *raf-1*, *lck*, and *ect2*. Other known genes included phospholipase C-γ_2, β-catenin, and the thrombin receptor. In addition to the known genes, seven novel cDNAs were isolated, including several members of the CDC24 family of guanine nucleotide exchange factors. Only the thrombin receptor was isolated more than once, and many of the 14 different genes identified were truncated within the protein coding region. The diversity of cDNAs isolated in the NIH3T3 assay is in contrast to results obtained in the current study. The specificity of the RK3E assay may be attributable to the "tumor suppressor" activity of the *E1A* oncogene (28, 45). Although *E1A* antagonizes $p105^{Rb}$ and immortalizes primary cells, it also induces epithelial differentiation in diverse tumor types, including sarcoma, and suppresses the malignant behavior of tumor cells *in vivo*.

GKLF was previously isolated by hybridization to zinc finger probes (30–32). The human gene is located at chromo-

some 9q31 and is closely linked to the autosomal dominant syndrome of multiple self-healing squamous epitheliomata (31, 32, 46, 47). Affected individuals develop recurrent invasive, but well-differentiated, tumors morphologically similar to squamous carcinoma that spontaneously regress. Although *GKLF* has been proposed as a candidate tumor suppressor gene relevant to multiple self-healing squamous epitheliomata (32), our results suggest that activating mutations could account for the syndrome.

GKLF encodes a nuclear protein that functions as a transcription factor when bound to a minimal essential binding site of 5′-$^{G}/_{A}{}^{G}/_{A}$GG$^{C}/_{T}$G$^{C}/_{T}$-3′ (48). The 470 residue polypeptide exhibits modular domains that mediate nuclear localization, DNA binding, and transcriptional activation or repression (31, 32, 49, 50). In mice, *GKLF* expression is found predominately in barrier epithelia, including mucosa of the mouth, pharynx, lung, esophagus, and small and large intestine (30, 32). A role for *GKLF* in differentiation or growth arrest was suggested by the onset of expression at the time of epithelial differentiation (approximately embryonic day 13; Refs. 32 and 51) and by similarity within the zinc finger domain to family members erythroid Krüppel-like factor and lung Krüppel-like factor that were previously associated with growth-arrest or differentiation-specific gene expression (52, 53). Similarity to these other genes is limited to the DNA-binding zinc finger region.

Our results show that *GKLF* can induce proliferation when overexpressed *in vitro*. Analysis of expression in dysplastic cells and tumor cells *in vivo* provides independent evidence that *GKLF* exhibits properties expected of an oncogene. Genetic progression of carcinoma seems to involve genes and pathways important for homeostasis of normal epithelium (6, 7, 9, 54). For example, the zinc finger protein GLI is expressed in normal hair shaft keratinocytes, whereas *c-MYC* is expressed in normal epithelium of the colonic mucosa. In tumors derived from these tissues, *GLI* and *c-MYC* are more frequently activated by recessive genetic changes in upstream components of their respective biochemical pathways than by gain-of-function alterations such as gene amplification. Up-regulation of *GKLF* expression in dysplastic epithelium and tumor cells *in vivo* is particularly interesting as expression seems not to be increased by proliferation *in vitro*. Expression of the endogenous *GKLF* mRNA in RK3E cells was similar in cycling *versus* contact-inhibited cells (data not shown). In contrast, *GKLF* is significantly induced in NIH3T3 cells during growth arrest (30). These different results suggest that cell type-specific mechanisms can regulate *GKLF* expression, and that *GKLF* may play different roles in epithelial *versus* mesenchymal cells.

Squamous epithelium is divided into compartments (55, 56). In the basal cell layer, proliferative reserve or stem cells possess long-term or unlimited self-renewal capacity, whereas the parabasal transit amplifying cells undergo several rounds of mitosis and then withdraw from the cell cycle to differentiate into spinous cells that form the mid strata of the epithelium. These cells then undergo terminal differentiation and programmed cell death at the surface. Proliferation and differentiation are normally balanced such that overall cell number remains constant. In contrast to *GLI* and *c-MYC*, *GKLF* expression in skin seems limited to the differentiating compartment (32). A simple model is that *GKLF* normally regulates the rate of maturation and shedding and the overall transit time for individual cells. The thickness of epithelium, which varies greatly in development and in different adult tissues, may be regulated not only by alterations in the rate of cell division in the basal layer, but also in response to *GKLF* or similarly acting molecules in the suprabasal layers. This model is consistent with the relatively late induction of *GKLF* during mouse development, and is testable by modulating expression of *GKLF* in transgenic animals or using raft epithelial cultures *in vitro*. Activation of *GKLF* in the basal layer of dysplastic epithelium suggests that dysplasia and progression to invasion and metastasis could result from loss of normal compartment-specific patterns of gene expression.

In summary, *GKLF*, *c-MYC*, and *GLI* are potent oncogenes in epithelioid RK3E cells *in vitro*, are analogous with respect to their expression in normal epithelium, and have potentially complex roles in the regulation of epithelial cell proliferation, differentiation, or apoptosis (6, 7, 9, 44, 56–58). How *GKLF* contributes to these processes will require a better understanding of its function and of the pathways that regulate *GKLF* activity in epithelia.

Materials and Methods

Immunocytochemistry. Immunocytochemical assays were performed in the Immunopathology Laboratory at The University of Alabama at Birmingham. Antibodies to vimentin and desmin were from Dako (Carpenteria, CA). A mixture of anticytokeratin included AE1/AE3 (Biogenics, San Ramon, CA), CAM5.2 (Becton Dickinson, San Jose, CA), and MAK-6 (Zymed, South San Francisco, CA). Human tissue served as a positive control for each antibody. No signal was obtained in the absence of primary antibody.

Construction of cDNA Libraries. Two cDNA libraries were constructed using the ZAP-Express cDNA synthesis kit (Stratagene, La Jolla, CA). A library was prepared from human squamous cell carcinoma cells derived from tumors of the oro-pharynx. Equal quantities of total mRNA from cell lines SCC15, SCC25, and FaDu (American Type Culture Collection, Manassas, VA) were pooled. Similarly, equal quantities of mRNA from the breast cancer cell lines MCF-7, ZR75–1, MDAMB-453, and T47D (American Type Culture Collection) were pooled. For each pool, poly(A)+ mRNA was selected by two cycles of oligo-dT cellulose affinity chromatography, and 5 μg were reverse transcribed using an oligo-dT linker primer and MMLV reverse transcriptase. Double-stranded cDNA was synthesized using *Escherichia coli* RNase H and DNA polymerase I. cDNA was ligated to λZAP EXPRESS bacteriophage arms and packaged into virions. The λ titer and the frequency of nonrecombinants were determined before amplification of the library on bacterial plates (Table 1). The frequency of nonrecombinant clones was estimated to be <2% by complementation of β-gal activity (blue/white assay). Phage were converted to pBKCMV plasmids by autoexcision in bacteria. Insert sizes in randomly selected clones were determined at this step by gel electrophoresis of plasmid DNA digested with *Sal*I and *Not*I (Table 1). The pBKCMV plasmid libraries were amplified in soft agar at 4×10^4 colony forming units/ml (27). After incubation at 37°C for 15 h, bacterial cells within the agar bed were isolated by centrifugation, amplified for 3–4 doublings in culture, and plasmid DNA was purified using a Qiagen column (Qiagen, Inc., Chatsworth, CA).

To generate libraries in a retroviral expression vector, cDNA inserts were excised from 10 μg of plasmid using *Sal*I and *Xho*I. After treatment with Klenow and dNTPs and extraction with phenol, the DNA was ligated to 5′ phosphorylated *Bst*XI adapters (5′-TCAGTTACTCAGG-3′ and 5′-CCTGAGTAACTGACACA-3′), as described (27). After treatment with *Not*I, excess adapters were removed by gel filtration, and the residual vector was converted to a 9.0-kb dimer using the *Not*I site and T4 DNA